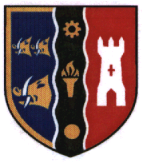

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Multiplex T-RFLP, a novel method to study rhizosphere-microbial interactions

Presented by

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Declaration

I declare that the work presented in this thesis is my own, except where otherwise acknowledged, and that this thesis was composed by me. All cited references are listed at the end of the thesis. No part of this thesis has been presented for any degree or qualification at any other academic institution.

Student Signature Date

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NAZARIES L. Multiplex T-RFLP, a novel method to study rhizosphere-microbial interactions.

Abstract

The rhizosphere is an active zone of the soil around the plant roots where important biotic interactions take place between plant roots and soil microorganisms. These interactions involve carbon cycling where recently assimilated atmospheric CO_2 is released by plants into the rhizosphere for microbial consumption, which in turn provides plants with other nutrients such as nitrogen and phosphorous. Most of the studies on rhizosphere-microbial interactions were performed with one group of microbes, *i.e.* Bacteria, Fungi or *Archaea* due to cost and timing involved in such analyses. Our study aim was to develop a reliable assay allowing the study of several microbial groups at once. We associated the features of the multiplex polymerase chain reaction (multiplex PCR), which can amplify several genes at once, with the sensitive detection of the terminal restriction fragment length polymorphism (T-RFLP) analysis, which is able to give information on the structure and composition of a given microbial community through production of characteristic fragmentation patterns. Primers for the eubacterial and rhizobial 16S as well as fungal ITS genes, which were 5'-labelled with three different dyes (respectively, VIC, PET and 6-FAM), were used for the multiplex PCR to amplify soil DNA. After digestion of the multiplex amplicons by restriction enzymes, the terminal restriction fragments (T-RFs) generated were separated and detected. A fragmentation pattern is characterised by the number, size and relative abundance of the T-RFs produced. The efficiency of the T-RFLP to detect fragments from multiplex PCR was compared with fragments from three separate individual PCRs (one for each gene marker). Our results showed a very high similarity (over 90 %) between the T-RFLP profiles obtained from both PCR conditions. Principal co-ordinate (PCO) analysis of T-RFLP profiles suggested a strong influence of the soil type (habitat) on the rhizospheric microbial community. The differences observed were independent of the plant species. The technique of multiplex T-RFLP proved to be a consistent and cost-effective method for the study of changes in the microbial community, and opens a wide range of applications in microbial ecology.

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Abbreviations

λ_{max}	maximum wavelength
bp	base pair
BSA	Bovine Serum Albumin
C	Carbon
CCD	Couple-Charged Device
CE	Capillary Electrophoresis
CLPP	Community Level Physiological Profiles
CO_2	carbon dioxide
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribo Nucleic Acid
dsDNA	double-stranded DNA
EDTA	Ethylene-Diamine-Tetra-Acetate
ITS	Internal Transcribed Spacer
kb	kilo bp
MiCA III	Microbial Community Analysis III
N	Nitrogen
N_2	atmospheric nitrogen
P	Phosphorous
PCO	Principal Co-Ordinate
PCR	Polymerase Chain Reaction
PEG	Polyethylene-Glycol
PLFA	Phospholipid Fatty Acid

POP-4	4 % (acrylamide) Performance Optimized Polymer
PVPP	Polyvinyl-Polypyrrolidone
RDP-II	Ribosomal Database Project II
RFU	Relative Fluorescence Unit
RNA	Ribo Nucleic Acid
rDNA/RNA	ribosomal DNA/RNA
SDS	Sodium Dodecyl-Sulphate
SOC	Soil Organic Carbon
SOM	Soil Organic Matter
spp.	species
ssDNA	single-stranded DNA
TAPS	N-Tris-(hydroxymethyl)methyl-3-aminopropane-sulfonic acid
TBE	Tris Borate EDTA
T-RF	Terminal Restriction Fragment
T-RFLP	Terminal Restriction Fragment Length Polymorphism

Chapter 1
Introduction

1.1 Background information

1.1.1 Interactions between plants and microbes within the rhizosphere

The rhizosphere is a biologically active zone of the soil around plant roots in which multitrophic processes are associated with nutrient cycling via the presence of plant roots coupled with the proliferation of microorganisms in the surrounding soil (Jones *et al.*, 2004). It is thus a zone of interactions of the plant roots with the soil microbes.

Plant-microbial interaction is vital for the proper functioning of an ecosystem. Plants provide carbon (C) to rhizospheric microbes, which in turn supply nitrogen (N), phosphorous (P) and other nutrients to plants by decomposition and immobilisation of the soil organic matter (SOM) (Singh *et al.*, 2004). In fact, a close interaction takes place in the rhizosphere between plants and soil microbes, which can be grouped under three categories:

- Pathogenic: when microbes are pathogens of the plant and derive benefits at the expense of the plant growth. Several fungal and bacterial diseases of plants are examples of negative interaction.
- Associative: both partners (the plant and the microorganism) are in loose association in which either both of them derive benefits or only one does but without harming the other. For example, plant growth can be enhanced by the presence of free-living N_2-fixing bacteria such as *Azotobacter* spp., which are

able to release ammonia and growth substances (*e.g.* auxins, cytokinins) and thus to increase the soil fertility (Mrkovački and Milić, 2001).

- Symbiotic: this is a type of association where both partners are physically associated and the close interaction is beneficial for both. Examples of symbiosis are mycorrhizae (Haselwandter and Bowen, 1996); and root nodules of legumes (Torrey, 1992) and non-leguminous plants (Faure-Raynaud *et al.*, 1990).

1.1.2 Ecosystem functioning and plant-microbial interactions

1.1.2.1 Rhizodeposition and root exudation

Rhizodeposition defines the total C released from plant roots into the surrounding soil. Different types of release may occur: water-soluble exudates (low molecular weight substances, which are lost passively), secretions (higher molecular weight substances, which are lost actively), lysates from dead cells, gases and mucilage (composed of polysaccharides) (Grayston *et al.*, 1996).

Root exudation corresponds to the release of small molecules such as amino acids, organic acids and sugars that are present in the cytoplasm at high concentrations (Farrar *et al.*, 2003). Factors associated with the plants (species, age and stage of development) and the environment (culture conditions, nutrient deficiencies, pH, soil humidity, light intensity, soil microorganisms) can influence the quality and quantity of root exudation in many ways. All these factors affect the quality and the quantity of root exudates. Therefore, it is important to understand the mechanism of rhizodeposition because it is a loss of carbon from the plant that has a significant impact on the soil microbial communities and hence on nutrient cycling (refer to section 1.1.2.2). Estimates of C loss due to rhizodeposition range from less than 10 to over 40 % of net C assimilation by the plant. The presence of symbiotic organisms increases the carbon translocation to the roots, which is usually compensated for by an increase in the photosynthetic rate (Grayston *et al.*, 1997).

Rhizodeposits, and thus root exudates, are important in the regulation of symbiotic and protective associations between plants and microorganisms. Root exudates have various functions and are involved in many processes. For example, the presence of flavonoids in root exudates of leguminous plants activates the *Rhizobium* genes responsible for the nodulation process (Peters *et al.*, 1986). The profile of flavonoids in root exudates is specific to the legume species and allows the symbiotic bacteria to distinguish their hosts from other legumes (Mithöfer, 2002). Root exudates also have antimicrobial properties and provide the plant with defensive advantages against soil-borne pathogens. Finally, rhizodeposits can change the chemical and physical properties of the soil in order to maintain a good root-soil contact, *e.g.* lubrication of the root tip or stabilisation of soil aggregates (Bais *et al.*, 2004).

1.1.2.2 Nutrient cycling and carbon sinks

Plant-microbial interactions in the rhizosphere have many consequences on the ecosystem functioning, especially on nutrient cycling as mentioned previously (section 1.1.1) with the role of the microbes in mineralization and immobilisation of the SOM (Singh *et al.*, 2004).

The soil microbial community also play an important role in carbon sinks and sequestration in the soil organic carbon (SOC) once the atmospheric carbon dioxide (CO_2) is metabolised and exuded in the soil by the plant roots (Zhu and Miller, 2003). Consequently, it is argued that the activity of the microbial communities, in tandem with the plants and associated sequestration of carbon, is an option to mitigate global climate change (Lal, 2003).

1.1.3 Microbial community structure in the rhizosphere

1.1.3.1 Factors affecting the composition of the rhizospheric microbial community

It was observed that the quantity and the quality of the organic matter provided by different plant species affect the microbial community (Wedin and Tilman, 1990). It was also shown that the plant species and soil depth influence the composition of this organic matter (Kuske *et al.*, 2002). Seasonal patterns of plant growth are known to affect soil microbial population size and biomass (Burke *et al.*, 2003). Microbial biomass is also increased in plant-species rich fields and nutrient-stressed plants due to augmented release of soluble carbohydrate in the root exudates (Chabrerie *et al.*, 2003).

The type of soil also influences the rhizospheric microbial composition. For example, successional gradient, *i.e.* conversion of one vegetation type into another, in chalk grassland, was found not to influence the microbial communities (Chabrerie *et al.*, 2003). Conversely, it was observed that modifications due to agricultural improvements (such as liming, fertilisation, increased grazing and reseeding) of the soil of an acidic grassland modify the soil fungal community structure rather than biomass (Brodie *et al.*, 2003). Finally, the composition of bacterial communities in grassland soils is influenced by the plant community composition rather than the plant species as well as by other factors such as soil characteristics (pH, water content) (Nunan *et al.*, 2005).

1.1.3.2 Constrains associated with the study on the microbial community structure

The studies of microbial diversity and community structure have been restricted until recently because only 1-10 % of the total community can be cultured in laboratory conditions (Torsvik *et al.*, 1998). Although the culture-based techniques provided useful information regarding microbial diversity in the rhizosphere, they suffered from bias resulting from the limitations of cultivability (Prosser, 2002). However, the development of improved physiological and biochemical techniques as well as novel molecular methods have allowed

assessment of the entire microbial community. Although the different techniques sometimes seem to give different results, it is argued that this is because they measure different components (Grayston *et al.*, 2004; Singh *et al.*, 2005). The most common and reliable techniques used are: community level physiological profiles (CLPP), phospholipid fatty acid (PLFA) profiles and DNA fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP).

CLPP is a physiological method based on the Biolog® assay that allows determination of the spectrum of carbon sources used by microbial communities extracted from soil. Analysis is achieved in a 96-well microtitre plate containing the substrates and a redox indicator (tetrazolium violet) to monitor the utilisation of carbon (absorbance at 590 nm) (Campbell *et al.*, 1997). Despite its limitation of cultivability, the culture-based approach of Biolog® allowed to observe differences in the rhizosphere microbial communities as well as the effects of external perturbations, such as the application of fertilisers, lime or sheep urine (Grayston *et al.*, 1998). Biolog® rapidly estimates the metabolic profile of a soil microbial community but fails to identify the microorganisms present.

PLFA is a biochemical method based on the unique fatty acid signature that certain groups of microorganisms possess (Vestal and White, 1989). After separation, identification and quantification by chromatography and mass spectroscopy, fatty acids can be grouped in broad categories representative of microbial communities. For example, the fatty acids 18:1ω9, 18:2ω6,9 are fungal biomarkers, and 16:1ω7, 18:1ω7 are Gram-negative bacterial PLFAs (Treonis *et al.*, 2004). PLFA provides a broad-scale diversity index such as the number of bacterial families present in a sample. However this method cannot offer a higher resolution of microbial diversity where it is necessary to identify key microbial species at the community level or to elucidate their role in the ecosystem.

The limitations of CLPP and PLFA have been overcome to some extent by the introduction of molecular techniques to analyse the microbial diversity using the ribosomal DNA (rDNA) as biomarker. Cloning and sequencing, as well as DGGE and T-RFLP, are molecular methods for DNA fingerprinting that provide very detailed and fine resolution of soil microbial community structure by analysing the genetic differences of each microbial population (Torsvik and Øvreås, 2002).

DGGE separates the PCR-amplified gene products of the same size but different sequences after chemical denaturation in a chemical gradient (Muyzer and Smalla, 1998). Thus, the separation of the different DGGE bands depends on the melting behaviour of the PCR products and not on the fragment size.

T-RFLP is an automated and sensitive method, which can be used to compare microbial communities and monitor changes in community structure (Marsh, 1999). A fluorescently labelled primer is used for the PCR, and after restriction digestion, fragments of different length are generated. Then the digested fragments are separated on the basis of their base pair size due to restriction site polymorphism (refer to section 2.5). The sequencer recognises only the fluorescently labelled terminal fragments and thus, each fragment represents a unique genome in the sample (Blackwood *et al.*, 2003).

Both DGGE and T-RFLP provided interesting results that helped understand plant-microbial interactions at both the taxonomic and functional levels (reviewed in Singh *et al.*, 2004). Yet, DGGE seems to have a lesser resolving power compared to T-RFLP (Nunan *et al.*, 2005).

1.2 Aim of the study

The understanding of nutrient cycling in soil is of prime importance for the comprehension of ecosystem functioning. It is now known that soil microbial communities play a vital role in nutrient cycling (Singh *et al.*, 2004). Environmental gradient such as moorland – pine forest (*Pinus sylvestris* L.) transition was found to influence soil fungal diversity (Anderson *et al.*, 2003). It was hypothesised by the authors that the presence of understorey plants like *Calluna vulgaris* can influence the fungal structure due to the ability of this type of plant to form ericoid mycorrhizas (Vrålstad *et al.*, 2002). Consequently, it is essential to understand the relationship between the plants and the microbes present in the surroundings of the plant roots, and between the different types of microorganisms.

Despite the advantage of higher resolution of molecular fingerprinting methods, these can only be used to study the diversity and structure of one taxonomic group or function at a time depending on the primers used for PCR. For example, fingerprinting methods (*e.g.* T-RFLP) can be employed to study structural diversity of microbes (such as bacterial diversity using 16S rDNA, fungal diversity using 18S rDNA or ITS) and functional diversity of ecosystems using nitrifying, denitrifying or methane oxidising genes. However, most of ecological studies require analysis of the structural and functional diversity of more than one taxonomic group. More importantly, studies on interactions between different microbial groups (Bacteria vs. Fungi, Fungi vs. *Archaea*) are vital to understand biotic interactions and ecosystem functioning.
This, in turn, needs the use of an increased number of individual marker genes followed by separate T-RFLP analysis for each gene, which is not only time-consuming but also very expensive. An integrated method allowing the analysis of more than one marker gene for structural diversity studies (such as bacterial, fungal, archaeal) or more than one taxonomic functional marker will be of great help to microbial ecologists to investigate more than one taxonomic group. This will consecutively give a better picture of microbial ecology of given ecosystems.

The aim of the study was to develop a novel molecular method, which can use more than one marker gene to study microbial diversity and community structure in soils. Another important aim was to examine if there were any differences in the structure of the rhizosphere microbial community of *Calluna vulgaris* at different growth stages and under different habitats such as forest, transition and moorland. For those purposes, the DNA fingerprinting technique T-RFLP was developed in association with a particular method of DNA amplification by PCR, the multiplex PCR. This novel technique is called **multiplex T-RFLP**.

Chapter 2
Materials and methods

2.1 Sample collection

2.1.1 Field site description

Field samples were obtained from an area of mature Scots pine (*Pinus sylvestris*) at the southern edge of Abernethy Forest, Cairngorm, Scotland (National Grid Reference NJ027122), extending in a south-easterly direction into open moorland. Within the intermediate zone there was active seedling colonisation by Scots pine that, at the time of sampling, were 15-20 years old, although growth was slow and few seedlings exceeded 1-1.3 m in height.

2.1.2 Root and soil sampling procedure

Samples were taken along three parallel transects (F, T and M). Each transect was divided into 10 sections with 11 points in total, **X** m apart. The first transect (F) was in the **forest** and the third one (M) in the **moorland**. The second transect (T) was situated in the **transition** region between F and M.

Calluna vulgaris was taken from all three transects (F, T and M) on each 11 points (F1C, F2C…F11C; T1C to T11C; M1C to M11C). *Vaccinum vitis-idaea* was only taken from the forest transect (F1V to F11V). This produced 44 field samples in total, of which only 43 had enough root materials for analysis. Roots were brought back to the laboratory and placed at -20˚C. Later, the samples were defrosted and for each one the fine hair roots were taken, along with the soil aggregated to them, but litter was avoided. The root+soil samples were then added to tubes containing a bead solution from the UltraClean™ Soil DNA Isolation Kit for the DNA extraction (refer to section 2.2.2).

2.2 DNA extraction from rhizosphere soil

2.2.1 Principle

The use of DNA for molecular techniques relies heavily on methods for the extraction of DNA. Hence the importance of collecting high quality nucleic acids as template. High DNA yields are also important to obtain a low detection limit and to ensure the DNA sample is representative of the soil gene pool.

Several techniques are available for the extraction of DNA from soils and sediments. Amongst them is the **cell extraction method** (Torsvik, 1980), which involves the separation of bacterial cells from the soil particles by differential centrifugation followed by lysis of the recovered cells, recovery of the DNA and its purification by hydroxyapatite column chromatography. Another important technique is the **direct lysis method** (Ogram *et al.*, 1988), which involves release of DNA from the cells by physical disruption followed by alkaline extraction of the DNA in buffer and its purification by ethanol precipitation, caesium chloride density gradient centrifugation and hydroxyapatite column chromatography.

The problem of further analysis of DNA from soils and sediments is the presence of co-extracted organic compounds such as **humic acids** (or humus). They are products from the decomposition of organic matter and thus have a very complex structure. They have a high power of chelating bivalent ions. Humic acids are also able to inhibit some restriction endonucleases (Steffan *et al.*, 1988) and to inhibit the PCR by inhibiting the *Taq* polymerase (Tsai and Olson, 1992) and by decreasing the efficiencies of DNA-DNA hybridisations (Steffan *et al.*, 1988). According to Tsai and Olson (1992), the humic acid effect can be attenuated by diluting the DNA solution but it also reduces the detection limit. Steffan and colleagues (1988) showed that both cell extraction and direct lysis methods can be improved by adding polyvinyl-polypyrrolidone (PVPP) (first used by Holben *et al.*, 1988), an insoluble polymer that is able to adsorb soil organic matter and remove it from cell preparations. Although using PVPP allows clean-up the DNA, humic acids will always be present and it can be useful to check the purity of the DNA by measurement of the ratio A_{260}/A_{230}

(humic acids absorbs UV light at 230 nm) for humic acid contamination. The ratio A_{260}/A_{280} for protein impurities can also be assessed. Higher yields of DNA are obtained with the direct lysis method (Steffan *et al.*, 1988).

The DNA extraction was later optimised by using a bead-beating method in a buffer, associated with a phenol-chloroform method for the DNA extraction and a polyethylene glycol (PEG) precipitation solution at high salt concentration (Bürgmann *et al.*, 2001). Yet, no PVPP was used due to the efficiency of using PEG to reduce the co-precipitation of humic compounds (Rowland and Nguyen, 1993).

2.2.2 Experimental approach

The total DNA was extracted from the soil samples using the UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, CA, USA) according to the manufacturer's instructions. The kit uses a method based on the direct lysis method coupled with the use of a PCR inhibitor removal solution.

Briefly, the tubes containing a bead solution were first used to dissolve the humic acids present and to disperse the soil particles. Then, DNA was released from the cells using a solution containing SDS (solution S1 of the kit) along with another solution (inhibitor removal solution – IRS) to precipitate the humic acids and other PCR inhibitors. For that step, the tubes were processed in a bead-beater (FastPrep FP120 bead-beater, Bio101/Savant, NY, USA) for 30 seconds at a speed of 5 $m.s^{-1}$, which is approximately 5000 rpm according to the manufacturer. After processing, the tubes were centrifuged at 10,000 x g for 1 minute and the supernatant was transferred into a fresh tube. After precipitation of the humic acids, proteins and cell debris (with solution S2), the supernatant was transferred into a fresh tube and mixed to a high salt-concentration solution (solution S3). The mixture was then centrifuged (10,000 x g for 1 minute) through a silica-membrane to which the DNA was able to bind. The DNA on the membrane was further washed with a solution containing ethanol (solution S4) to remove residues of salt, humic acids and other contaminants (centrifugation at 10,000 x g for 30 seconds). Finally, the DNA was eluted from the silica filter into a fresh tube with 50 µl of 10 mM Tris buffer pH 8 (solution S5) by centrifugation at 10,000 x g for 30 seconds.

Because amplification from the DNA extracted was weak, further clean-up of the DNA samples was performed according to a classical DNA precipitation method: the DNA was precipitated with 3 parts of 100 % ethanol containing 3 M of sodium acetate (pH 4.6). The mixture was left at room temperature for 3 minutes and centrifuged (30 minutes at 12,000 x g) to collect the DNA. The supernatant was discarded and the DNA was washed with 150 µl of 70 % ethanol. The mixture was centrifuged for 30 minutes at 12,000 x g to precipitate further the DNA and the supernatant was discarded. After air-drying the pellet, DNA was resuspended in 30 µl of sterile Millipore Q water.

Soil DNA samples (43 in total) were stored at -20˚C until further use, *i.e.* PCR.

2.3 Polymerase Chain Reaction (PCR) and multiplex PCR for the detection of microbial communities

2.3.1 Principle

The extraction of total DNA from soil samples provides biological material without the need to isolate microorganisms by cultivation. Thus the analysis of directly extracted DNA has the potential to detect specific genes or to identify non-cultivable microorganisms.

The polymerase chain reaction (PCR) has the capacity to amplify very specific sequences of DNA. Since its introduction by Saiki *et al.* (1985 and 1988), it can be used to isolate (and amplify) a target DNA fragment from a mixture of different DNAs by using a pair of DNA templates – called primers – of specific sequence. These primers are usually about 20 bases long with a sequence complementary to the sequence immediately adjacent to the DNA segment of interest (one primer for each strand of the DNA, which binds either side of the sequence to be amplified). The sequence between the two primers is copied by using a DNA polymerase (*Taq* DNA polymerase – extracted from the organism *Thermus aquaticus* capable of sustaining temperatures above 60˚C) in the presence of a mixture of the four free deoxynucleotides (dATP, dTTP, dCTP and dGTP). Several cycles of PCR are needed to obtain an exponential multiplication of the target DNA sequence. Each PCR cycle is divided in three steps of specific temperature: first is the denaturation step at 95˚C, which separates the two stands of the double-stranded DNA (dsDNA) molecule. Then during the annealing step at 55˚C, each primer binds to its complementary sequence on the single-stranded DNA (ssDNA). Finally, there is the extension step at 72˚C: the *Taq* polymerase enzyme polymerises the complimentary DNA strand of each ssDNA from the primers. This cycle is then repeated a number of time. The target DNA synthesized is amplified a million times in 20 cycles, or a billion times in 30 cycles, which can be done in a few hours.

The temperatures for each step of the PCR can be changed in order to optimise the DNA amplification. Many other parameters can be altered, such as the buffer concentration and its $MgCl_2$ content, as well as the primers, *Taq*

polymerase and deoxynucleotides concentrations. It has been proved that the addition of non-acetylated bovine serum albumin (BSA) to the PCR reaction mixture offers some relief of inhibition from PCR inhibitors such as humic acids (Kreader, 1996). On the other hand, using acetylated BSA in a PCR buffer inhibits amplification (McKeown, 1994).

Multiplex PCR is a variation of PCR, which enables the simultaneous amplification of many DNA targets of interest in one reaction by using more than one set of primers.
Since its first description (Chamberlain *et al.*, 1988), this method has been applied in many areas of DNA testing, including analyses of deletions, point mutations (Manam and Nichols, 1991), and polymorphisms. Typically, it is used for genotyping applications where simultaneous analysis of multiple markers is required, detection of pathogens (Wilton and Cousins, 1992) or genetically modified organisms (GMOs). Multiplex assays can be time-consuming to establish, requiring lengthy optimisation procedures.

2.3.2 Experimental approach

2.3.2.1 Primer design

The primers used for both the individual and multiplex PCRs were the same. All the primers were obtained from Qiagen Operon (Qiagen, Cologne, Germany) and Applied Biosystems (Foster City, CA, USA).
The primer 1087r was targeting the universal eubacterial domain of the 16S rDNA. The primer 1244r was designed to indicate members of the *Rhizobium* and *Agrobacterium* species from the bacterial domain by targeting a more specific section of the 16S ribosomal RNA (rRNA) gene. The counterpart forward primer for the two reverse primers mentioned before was the 63f, universal primer for the 16S. The primers ITS1f and ITS4r were used to amplify the fungal-specific ITS (Internal Transcribed Spacer) region within the 18S rDNA. The expected PCR product sizes were ~1000 bp and ~1200 bp for the bacterial and rhizobial/agrobacterial 16S, respectively; and ~600 bp for ITS

although several bands were anticipated due to the variation of the gene size between the different fungal species.

Three sets of primers were then used to detect three genes. Within each set, one of the primers was labelled on the 5'-end with a fluorescent marker to allow 5'-terminal fragment detection and analysis after the T-RFLP assays (refer to sections 2.5 and 2.6). Primer 1087r was labelled with the marker VIC® (green colour code; refer to section 2.6.1); primer 1244r with marker PET™ (red); and primer ITS1 with 6-FAM™ (blue). The Table 2.3-1 shows the sequences of the different primers used for the different PCR experiments.

Primer name	Fluorescent label	Sequence from 5' to 3'	Target gene	Specificity
63f	none	AGGCCTAACACATGCAAGTC	16S rRNA	all Bacteria
1087r	VIC (green)	[VIC]-CTCGTTGCGGGACTTACCCC	16S rRNA	all Bacteria
1244r	PET (red)	[PET]-CTCGCTGCCCACTGTCAC	16S rRNA	*Rhizobium* and *Agrobacterium* spp. only
ITS1f	6-FAM (blue)	[6-FAM]-CTTGGTCATTTAGAGGAAGTAA	ITS (18S rRNA)	all Fungi
ITS4r	none	TCCTCCGCTTATTGATATGC		

Table 2.3-1: Primers used for the individual and multiplex PCRs.

2.3.2.2 PCR and multiplex PCR amplification conditions

The PCR mastermix (50 µl final reaction volume) contained 1X NH_4 reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.01% Tween-20], 2 mM $MgCl_2$, 250 µM of each dNTP (dATP, dTTP, dCTP and dGTP), and 2.5 units of Biotaq DNA polymerase (all reagents from Bioline, London, UK). Non-acetylated BSA (Roche Diagnostic, Lewes, UK) was also added at a concentration of 400 $ng.\mu l^{-1}$. At first, it was decided to use the same concentration of each primer, but amplification of the ITS gene was very faint and the ITS primer concentration was then doubled. Finally, it was decided to use 10 $pmol.\mu l^{-1}$ of each of the bacterial and rhizobial primers (63f, 1087r and 1244r) and 20 $pmol.\mu l^{-1}$ of the fungal primers (ITS1 and ITS4) for the individual PCRs. When performing a multiplex PCR, the concentrations of primers were the same but less (sterile Millipore Q) water was used to complete the mastermix volume to 48 µl. Once the mastermix was ready, it was aliquoted

into 200 µl-tubes and 2 µl of DNA sample was added. The tubes were then placed in the thermocycler and the amplification performed. Positive and negative controls were included in each PCR: positive controls were DNA from bacterial and fungal isolates when amplifying 16S and ITS fragments, respectively. When the rhizobial gene was amplified, a community DNA was used. For negative controls no DNA template was added.

Amplification of all three target sequences for both individual and multiplex PCRs was performed with a DYAD™ DNA Engine® Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) using the same program for all conditions, *i.e.* for the PCRs with each primer as well as when multiplexed. The sole PCR program employed was a typical 16S PCR amplification program consisting of a primary step of 5 minutes at 95°C followed by 30 cycles including a denaturing step at 95°C for 30 seconds, an annealing step at 55°C for 30 seconds and an elongation step at 72°C for 1 minute. The last cycle was followed by incubation at 72°C for 10 minutes to allow elongation of any remaining DNA and make sure that no single strands of DNA were left.

To avoid any contamination, the PCR tubes were UV-irradiated, before the PCR reaction, using a UV-crosslinker (CL-1000 model, UVP, Cambridge, UK) for 2 minutes at 2 $mJ.cm^{-2}$.

After PCR, amplicons were kept at -20˚C. They were later cleaned-up to improve their level of purity.

All 43 soil DNA samples were amplified with each three primer sets (for 16S, ITS and RHIZ [*Rhizobium* spp.]) individually and mixed together (multiplex PCR). 172 PCR samples (43*3+43) were then obtained.

2.3.2.3 Detection of amplified DNAs

After amplification, size and purity of the PCR products (or **amplicons**) were confirmed on an agarose gel. 5 µl of loading buffer (0.25 % bromophenol blue, 40 % sucrose) was added to 5 µl of the PCR products and to 5 µl of molecular weight marker (HyperLadder I from Bioline). The resulting 10 µl were loaded on a 1 %-agarose gel (w/v) (0.75 cm-thickness) stained with 1 µl of 10 $mg.ml^{-1}$ ethidium bromide (Sigma-Aldrich, Dorset, UK). The gel was run in 1X TBE (Tris Borate EDTA) buffer for 20 minutes (individual PCR) or 45 minutes (multiplex

PCR) at 100 Volts using a BioRad electrophoresis tank coupled to a BioRad powerpack (Bio-Rad Laboratories, Hercules, CA, USA).

Both agarose gel and running buffer used 1X TBE diluted from a 5X TBE buffer containing 450 mM Tris base (Promega, Madison, ME, USA), 450 mM Boric acid (Promega) and 10 mM EDTA (pH 8) (Promega). The agarose was from Cambrex (Rockland, ME, USA).

After migration, the gel was observed under UV light (365 nm) on a Gene Genius UV analyser (SynGene, Cambridge, UK) coupled to the Gene Snap software (version 4.01.00, SynGene).

The molecular weight markers profile of the HyperLadder I consisted of fourteen bands between 200 and 10,000 bp (Figure 2.3-1).

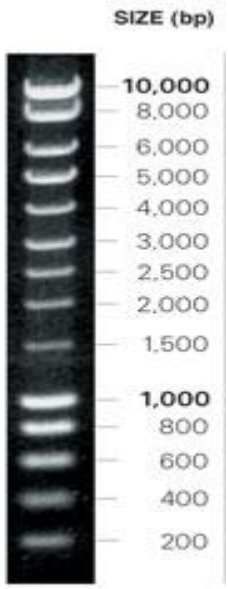

Figure 2.3-1: HyperLadder I migration profile in 1 % agarose gel.
The higher intensity bands are 1 and 10 kb. From Bioline.

2.4 Purification of PCR products

2.4.1 Principle

After a PCR reaction, many reagents are present in the tubes. Excess of primers, nucleotides, DNA polymerase, oil and salts can greatly affect the purity of the amplified DNA. It can later affect the performance of the DNA fragment analysis. It is therefore necessary to clean-up the PCR products.

The principle of amplicon purification is the same as in the DNA extraction procedure detailed previously when using the UltraClean™ Soil DNA Isolation Kit (refer to section 2.2.2): DNA present in the solution binds to a silica membrane when a high-salt concentration solution is applied; DNA is washed with an ethanol-based solution and then eluted using a low-ionic strength buffer such as TE (Tris-EDTA), pH 8.5 or 10 mM Tris-HCl, pH 8.5.

PCR fragments are now ready for a variety of manipulations including restriction analysis, ligation, sequencing, or labelling.

2.4.2 Experimental approach

The PCR products were purified using the GenElute™ PCR clean-up Kit (Sigma-Aldrich, Dorset, UK) according to the manufacturer's instructions. The kit is designed for rapid purification of single-stranded or double-stranded PCR amplification products (100 bp to 10 kb). This kit also eliminates the need for expensive or toxic organic compounds such as phenol or chloroform.

Briefly, the GenElute Miniprep Binding Column was first rehydrated using the Column Preparation Solution (centrifugation at 12,000 x g for 1 minute). One volume of the PCR reaction (maximum of 100 µl) was mixed to five volumes of the Binding Solution. The mixture was centrifuged (14,000 x g for 1 minute) through the Binding Column and the flow-through liquid was discarded. The PCR fragments were washed with the ethanol-diluted Wash Solution (centrifugation at 14,000 x g for 1 minute) to remove contaminants. The tubes were further centrifuged (2 minutes at 14,000 x g) to remove excess of ethanol. The column was transferred into a fresh tube and 50 µl of the Elution Solution

(10 mM Tris-HCl, pH 8.5) was applied on the silica membrane for 1 minute at room temperature. DNA was finally eluted by centrifugation at 14,000 x g for 1 minute.

Cleaned amplicons (172 PCR samples) were stored at -20˚C until further utilisation, *i.e.* DNA measurement and T-RFLP analysis.

2.5 Terminal Restriction Fragment Length Polymorphism (T-RFLP) of the PCR products

2.5.1 Principle

T-RFLP is a culture-independent DNA fingerprinting method that can be used to determine the structure and diversity of microbial communities. By coupling it to the PCR technique, T-RFLP allows the characterisation of uncultivable microbial species.

It was first used to identify the specific bacterial species within a complex mixture by studying the variability of the small subunit rRNA (SSU rRNA) genes (Avaniss-Aghajani *et al.*, 1994). The T-RFLP technique involves a pre-PCR step: first, it requires tagging one end of the PCR products through the use of a fluorescent molecule (or **fluorochrome**) attached to one of the primers at the 5'-end. The amplified products are then cut with a restriction enzyme. Terminal restriction fragments (T-RFs) are separated by electrophoresis according to their size, and visualised by excitation of the fluorochrome by a laser beam. The output is analysed by specific softwares and the results (peaks) are presented as an **electropherogram** (refer to section 2.6.1).

The nucleotide sequence of the 16S rRNA gene is unique for each bacterial genome. Consequently, once the fragment has been cut with a restriction enzyme, the fluorescently labelled restriction fragments from different bacterial species often have characteristic lengths. Thus, the different fluorescent peaks that appear in a capillary electropherogram correspond to labelled restriction fragments from different bacterial genomes. Therefore, this protocol allows identification of a number of different bacterial genomes in a complex mixture (Liu *et al.*, 1997).

2.5.2 Experimental approach

Prior to digestion, purified PCR product concentrations were determined spectrophotometrically (UV photometer, Eppendorf, Germany). Initially, four different enzymes (*Hae* III, *Hha* I, *Msp* I and *Rsa* I) were used on four randomly

selected samples to determine the best enzymes for further analysis. *Hha* I and *Msp* I produced more peaks and gave higher consistency. Therefore, all samples were only digested with *Hha* I and *Msp* I. Different quantities of digested DNA (1000, 500 and 200 ng) were also tested: 500 ng gave the best results.

In a 20 µl-reaction mixture, 500 ng of DNA were added in a solution containing 1X enzyme buffer, 0.1 $\mu g.\mu l^{-1}$ of acetylated BSA and 20 units of restriction enzyme (all reagents from Promega). The two restriction enzymes were used with their corresponding buffer. The Table 2.5-1 shows the characteristics of each enzyme.

Enzyme name	Organism of origin	Restriction site	Optimum activity temp.	Enz. buffer used	Buffer composition
Msp I	*Moraxella* spp.	5'-C˅CGG-3' 3'-GGC˅C-5'	37˚C	10X buffer B	60 mM Tris-HCl (pH 7.5) 500 mM NaCl 60 mM $MgCl_2$, 10 mM DTT
Hha I	*Haemophilus haemolyticus*	5'-GCG˅C-3' 3'-C˅GCG-5'	37˚C	10X buffer C	100 mM Tris-HCl (pH 7.9) 500 mM NaCl 100 mM $MgCl_2$, 10 mM DTT

Table 2.5-1: Characteristics of the enzymes used for the T-RFLP
The sign ˅ indicates where the enzyme cuts the DNA fragment.

Samples were incubated at 37˚C for 3 hours followed by 15 minutes at 95˚C to stop the reaction. The thermocycler used was the same as for the PCR.

The T-RFLP experiments were performed according to **three approaches**. The first one was the digestion of 500 ng of DNA from all 43 samples from each individual PCR (*i.e.* with each individual primer set for 16S and ITS; for RHIZ, only 200 ng of DNA was used) with each restriction enzyme *Hha* I and *Msp* I. That, in total, gave 258 T-RFLP samples (43*3*2). Another approach for the T-RFLP experiments consisted of mixing together DNAs from each individual PCR (200 ng of DNA from amplification with 16S primers, 200 ng from ITS and 100 ng from RHIZ) and performing the digestion with each enzyme. That was another 86 T-RFLP samples (43*2). Finally, for the last approach, all 43 samples from the multiplex PCRs were digested with each enzyme using 500 ng of DNA, which gave 86 samples. Thus, a total of 430 samples (258+86+86) were analysed by T-RFLP and later submitted for fragment analysis.

2.6 Fragment analysis from the T-RFLP experiment

2.6.1 Principle

Once the DNA is digested by the restriction enzyme(s), it is run on a sequencer to gather information on the size of the different terminal fragments obtained. Since the restriction site of the enzyme is always the same but can be found at different loci of different microbial species, unique and characteristic patterns can be found. The genetic sequencer ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) can be used for this purpose. It uses the technique of capillary electrophoresis (CE) to separate fluorescently labelled DNA fragments. The detection of the fluorescence enables sizing and quantification of the DNA fragments.

Prior to analysis, samples must be denatured by heating at 95˚C in the presence of formamide, a denaturing agent. Then, fluorescently labelled DNA is introduced into a capillary by electrokinetic injection, *i.e.* a current is applied and the negative ions are sucked into the capillary. The capillary is internally uncoated (to suppress electro-osmotic flow) and filled with a flowable polymer that allows separation of the DNA fragments according to their size. The DNA fragments move within the capillary and when they reach the detection window at the end of the capillary, the dye molecules are excited by an argon ion laser beam and fluoresce. The (total) fluorescence emissions are then focused on a diffraction grating. The resulting light spectrum is separated by wavelength, focused onto a couple-charged device (CCD) camera and collected by a virtual (emission) filter set at the corresponding dye maximum emission wavelength. Then, the data collection software interprets the fluorescence data.

A (fluorescent) **internal size standard** needs to be added to each sample to estimate the sample fragment size. Several dyes can be used at the same time. Thus different labelled samples can be electrophoresed simultaneously. When using a dye set with five colours (*e.g.* dye set DS-33), four samples and an internal size standard can be electrophoresed at once. The different dyes have different fluorescent light intensity in non-overlapping regions, *i.e.* they fluoresce at different wavelengths.

Once collected, the fluorescence intensity data are colour-coded according to their maximum emission wavelength and displayed as coloured peaks in an electropherogram. The Table 2.6-1 shows the characteristics of the fluorescent dyes present in the dye set DS-33 when using the virtual filter set G5.

Dye	Excitation λ_{max} (nm)	Emission λ_{max} (nm)	Colour code
6-FAM	494	517	blue
VIC	538	552	green
NED	553	575	yellow
PET	558	595	red
LIZ	638	655	orange

Table 2.6-1: Characteristics of the fluorescent molecules of the dye set DS-33.

The Figure 2.6-1 represents a hypothetical electropherogram of a fragment analysis performed with the G5 filter/dye set.

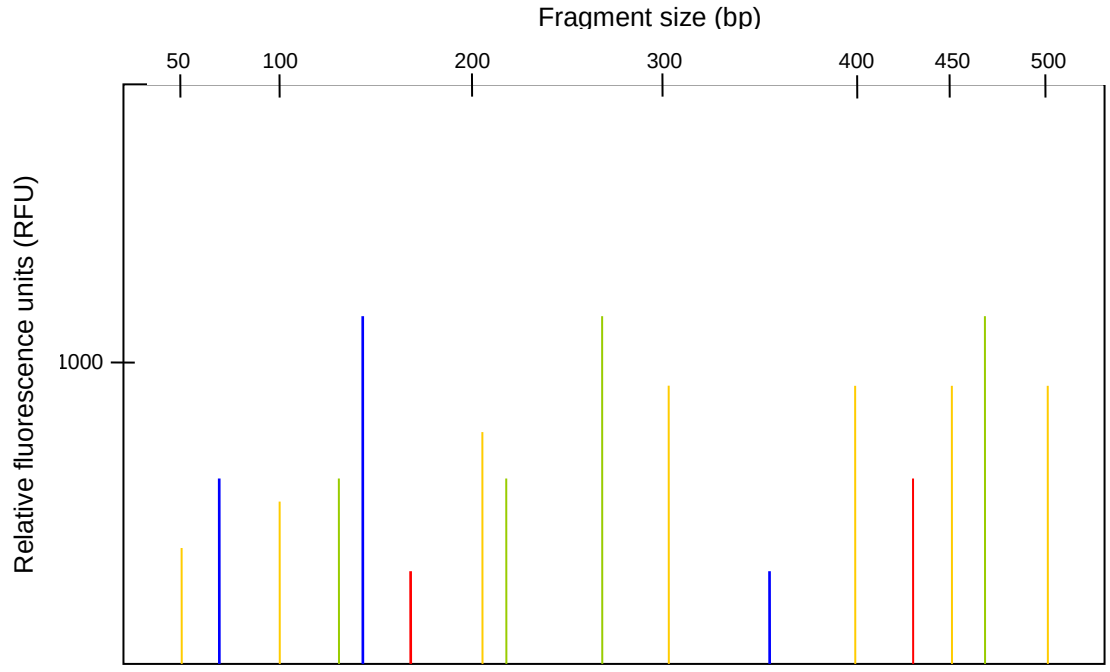

Figure 2.6-1: Hypothetical electropherogram with 3 colours (6-FAM in blue, VIC in green and PET in red) and the internal size standards (LIZ in orange)
The blue display represents the total light intensity from the shortest wavelength range monitored and the red display represents the total light intensity from the longest wavelength range monitored. The same colours are used to colour-code fluorescence data from all dye/virtual filter set combinations. Thus, the display colours represent the relative, not the actual, detection wavelengths. For example, the LIZ dye is not orange but deep red.
There can be any number of virtual filters, since the filter is simply a software-designated site on the CCD array.

2.6.2 Experimental approach

In this study, the T-RFLP technique was employed to identify three targets: the rRNA genes of bacteria and *Rhizobium*/*Agrobacterium* spp. (16S gene), and fungi (ITS gene) communities. The PCR primers were labelled at the 5'-end with three different types of dyes: primer 1087r was tagged with the molecule VIC®, primer ITS1f with 6-FAM™ and primer 1244r with PET™. The amplicons from the multiplex PCR therefore gave three types of coloured peaks after T-RFLP analysis of the samples and a fourth colour (orange) corresponded to the internal size standard LIZ.

After T-RFLP, 2 µl of the restricted PCR products was transferred to a MicroAmp optical 96-well reaction plate (Applied Biosystems) and mixed with 0.3 µl of LIZ-labelled GeneScan™-500 internal size standard (35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 bp) and 12 µl of Hi-Di™ (highly deionised) formamide (all reagents from Applied Biosystems). Prior to fragment analysis, samples were denaturated at 95˚C for 5 minutes and chilled on ice for 5 minutes. After a short centrifugation of the 96-well plate, DNA fragments were processed on the ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems) equipped with sixteen 50 cm-capillaries (50 µm internal diameter) filled with POP-4™ (4 % poly-dimethylacrylamide) polymer (Applied Biosystems). The fluorescently labelled DNA was loaded into the capillaries and the DNA fragments were separated according to their size. The running buffer used for the CE was 1X Genetic Analyser Buffer with EDTA (100 mM TAPS, 1 mM EDTA [pH 8.0], from Applied Biosystems). The Table 2.6-2 shows the settings used for the fragment electrophoresis.

Oven temperature	60˚C
Injection voltage	1.6 kVolts
Injection time	15 seconds
Running voltage	15 kVolts
Running time	2,500 seconds
Column	capillaries 16x50 µmx50 cm
Filter set	G5 dye set (FAM, VIC, NED, PET, LIZ)

Table 2.6-2: Settings of the ABI PRISM® 3130*xl* for the analysis of the T-RFLP fragments.

When the DNA fragments reached the detection window of the capillaries, the LIZ-labelled size standards and the 5'-terminal fragments were visualised by excitation of the fluorochromes (6-FAM™, VIC® and PET™ – all part of the G5 dye set from Applied Biosystems) attached to the 5'-end of the forward or reverse primers (respectively, ITS1f, 1087r and 1244f). Excitation (at 488 and 514.5 nm) was performed using an argon ion laser beam. Fluorescence data were analysed by the Data Collection software (version 3.0, Applied Biosystems). Terminal restriction fragment (T-RF) sizes and quantities were analysed with the GeneMapper software (version 3.7, Applied Biosystems) using the advanced peak detection algorithm. T-RF size (in base pairs) and intensity (peak height in relative fluorescence unit [RFU]) were estimated by reference to the internal standard. A binary table for presence and absence of peaks was made from these data for further statistical analysis.

This protocol was repeated for each of the three T-RFLP approaches, that is, digestion of the amplicons from the **individual, mixed and multiplex PCRs**.

2.7 Statistical analysis

2.7.1 Data processing

T-RFLP profiles were analysed with the GeneMapper software. T-RFs were quantified by advanced mode using second order algorithm. The relative abundance of a detected T-RF within a given T-RFLP profile was calculated as the respective signal height of this peak divided by the total peak height of all the peaks of the T-RFLP pattern. Only peaks between 50 and 500 bp were considered to avoid T-RFs caused by primer-dimers and to stay within the linear section of the size standards. Any peak which height was less than 0.5 % of the total peak fluorescence of the profile was excluded from further analysis.

2.7.2 Similarity matrix

A peak-by-peak comparison was carried out between profiles of same samples obtained by different methods of PCR and sequencing, *i.e.* individual PCR, multiplex PCR and mixed sequencing.

T-RFLP profiles were aligned by inspection of the electropherogram and by manual grouping of the peaks into categories. Alignment of peaks by manual inspection was based primarily on the size of peaks in base pairs, although the pattern of peaks was also used to determine their alignment when groups of overlapping peaks were found between samples. Once the manual alignment of the peaks performed, the presence of each fragment was compared between the different conditions (individual and multiplex PCR and mixed sequencing) and the similarity was calculated by dividing the number of similar peaks by the number of peaks present in the sample with the lowest peak number.

The above manual alignment was carried out for the analysis of the bacterial 16S and ITS fragments only. Analysis of the rhizobial 16S fragments consisted of the detection of the presence or absence of the predicted peaks. Predicted peaks were obtained from a web-based database search through the Microbial Community Analysis III (MiCA III) website at the University of Idaho (http://mica.ibest.uidaho.edu/trflp.php) on the basis of the primer sequence and the restriction enzymes used.

2.7.3 Statistical tests

Compiled data obtained from T-RFLP analysis were exported to Excel (Microsoft, Redmond, WA) to be converted into binary data (presence or absence of individual peaks). Binary data were then exported to GenStat 8th edition (VSN International Ltd, Hempstear, UK) for principal co-ordinate (PCO) analysis. PCO analysis was used to identify underlying patterns in the similarity data by deriving PCO scores from the samples.

PCO analysis of T-RFLP was chosen because the Jaccard similarity matrix is appropriate for binary data where the absence of a peak in both samples is less indicative of similarity than the presence of a peak in both samples. PCO analysis was used as multivariate function of GenStat using replicates as factors.

Chapter 3
Results

3.1 Optimisation of the methodology

Three approaches were chosen for the T-RFLP analysis based on the amplification of three microbial groups using different sets of primers:

- Digestion with each enzyme of the PCR products from the **individual PCRs**, *i.e.* the PCRs performed using only one set of primers.
- Digestion with each enzyme of the mixture of the PCR products from the individual PCRs, *i.e.* the three types of amplicons are present but come from three different reactions. It is important to note that the PCR products used in this approach (**mixed PCRs**) are the same that those obtained from the individual PCRs and used in the previous approach.
- Digestion with each enzyme of the PCR products from the **multiplex PCRs**, *i.e.* the PCRs performed using the three sets of primers in the same amplification reaction.

Both PCR and T-RFLP methods needed to be optimised.

3.1.1 PCR optimisation

3.1.1.1 PCR conditions

The first step was to make sure that the sole PCR program used (refer to section 2.3.2.2) could amplify each gene properly to gather enough DNA for the different analyses.

Samples were taken randomly from the different transects and individual PCRs were performed using the different sets of primers. The amplicons were run on a 1 %-agarose gel to visualise the bands obtained (Figure 3.1-1).

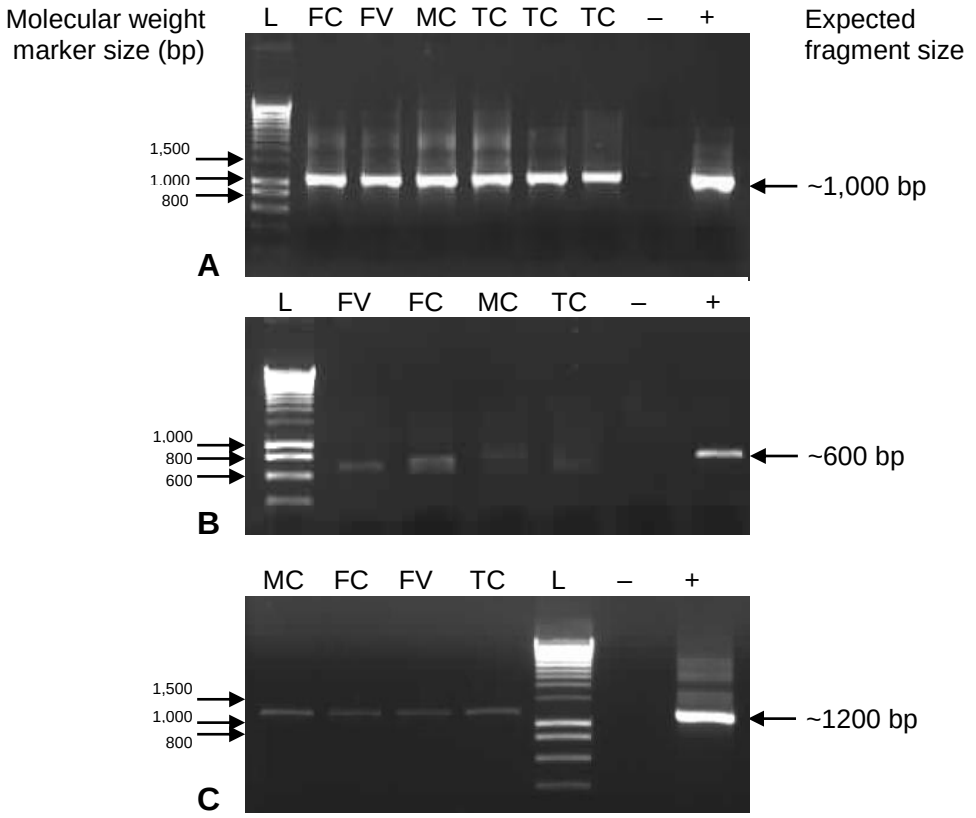

Figure 3.1-1: Amplification of the bacterial 16S (A), ITS (B) and rhizobial (RHIZ) 16S (C) genes.
Legend: L= Ladder, FC = Forest transect of *Calluna vulgaris*, FV = Forest transect of *Vaccinum vitis-idaea*, TC = Transition transect of *C. vulgaris*, MC = moorland transect of *C. vulgaris*
– = negative control, + = positive control.
Positive controls were bacterial (A), fungal (B) and community (C) DNAs. Negative controls did not contain DNA.

The PCR program was able to amplify DNA with the three sets of primers although amplification of the ITS gene (Figure 3.1-1B) was faint. The ITS primers concentration was later optimised.

3.1.1.2 ITS primer concentration

In order to augment the quantity of the fungal DNA amplified, the ITS primer concentration was increased by two folds compared to the concentration in the previous reaction.

Random samples were amplified with two different concentrations of the ITS primers: 10 and 20 pmol.µl^{-1} (see Figure 3.1-2).

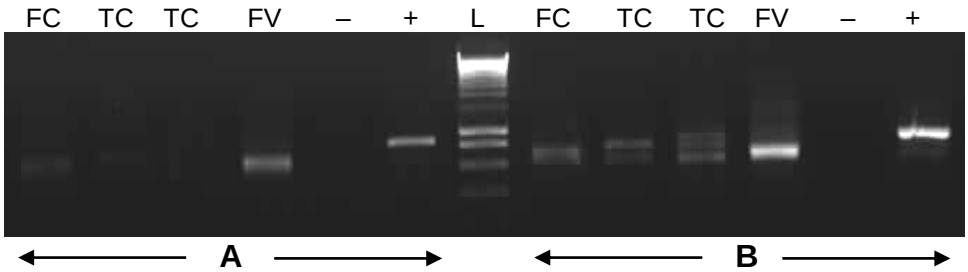

Figure 3.1-2: Optimisation of the ITS gene amplification using 10 pmol.µl^{-1} (A) and 20 pmol.µl^{-1} (B) of primers.

Although the bands for the sample FC and TC were faint, a better amplification of the ITS gene was generally obtained when using 20 pmol.µl^{-1} of the fungal primers (Figure 3.1-2B).

3.1.1.3 Assessment of the multiplex PCR

Random samples from each transect were amplified by individual and multiplex PCR according to the set protocol (refer to section 2.3.2.2).

The Figure 3.1-3 shows the migration patterns of the samples after multiplex and individual PCR amplification.

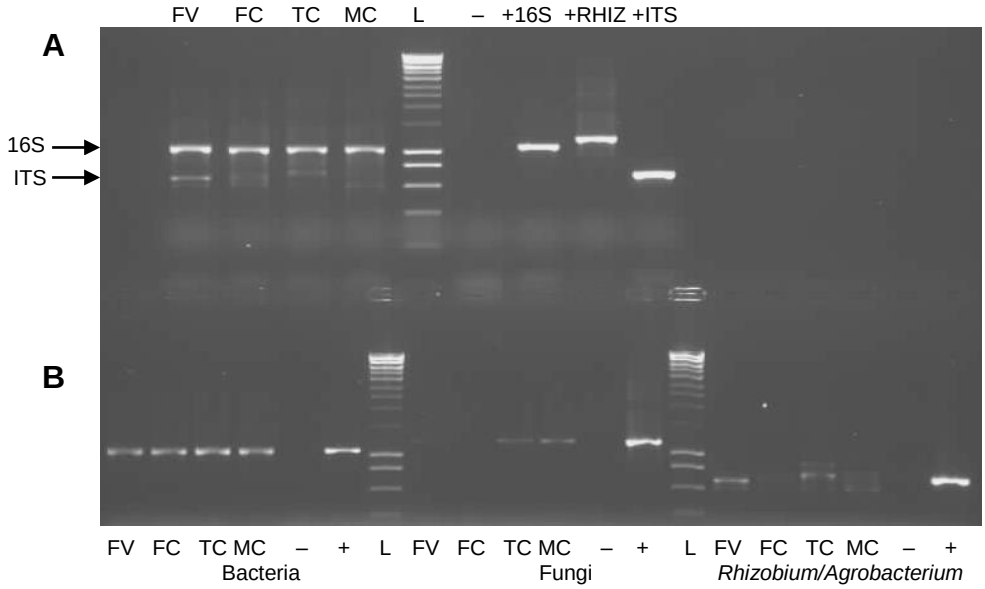

Figure 3.1-3: Multiplex (A) and individual (B) PCRs of the 16S, ITS and RHIZ genes. +16S, +ITS and +RHIZ were the positive control of the multiplex PCR for bacteria, fungi and *Rhizobium/Agrobacterium*, respectively. They were the same as for the individual PCR except they were amplified with the three sets of primer present.

The multiplex PCR (Figure 3.1-3A) allowed the amplification of the three genes in the samples. The 16S and ITS bands were present but the RHIZ band was not visible.
Note: the lack of amplification observed in the individual PCRs (especially for fungi FV and FC and for *Rhizobium/Agrobacterium* FC, Figure 3.1-3B) was due to the presence of humic acids in the sample.

3.1.2 T-RFLP optimisation

3.1.2.1 Restriction enzyme

Initially, several enzymes were tested in order to choose two that would give the best peak (restriction fragment) pattern.
Four samples were randomly chosen and amplified by multiplex PCR. The products were purified and digested for 30 minutes only with the four following restriction enzymes: *Hae* III, *Hha* I, *Msp* I and *Rsa* I. The Figure 3.1-4 represents the T-RFLP profiles observed with each enzyme.

Results showed the enzyme *Rsa* I did not produce evenly distributed peaks of the ITS gene (blue) with no peaks of the RHIZ gene (red) and only a few peaks clustered at ~210 bp were present for 16S (green). Conversely, the other enzymes produced many more peaks but those obtained with *Hae* III were mostly under 200 bp with some off-scale heights and the peaks were not consistent between the samples (data not shown). But *Hha* I and *Msp* I produced nice peaks with a good range of fragment sizes for fungi and bacteria. The RHIZ gene (rhizobial 16S) was always present in low quantity (very low peaks) and hidden by the other bigger peaks. When an individual PCR of the RHIZ gene was analysed by T-RFLP using *Hha* I and *Msp* I (Figure 3.1-5), three peaks were observed: two peaks (156 and 158 bp) for *Hha* I digestion and one peak (87 bp) for *Msp* I digestion.

Therefore, the enzymes *Hha* I and *Msp* I were chosen for all further analyses.

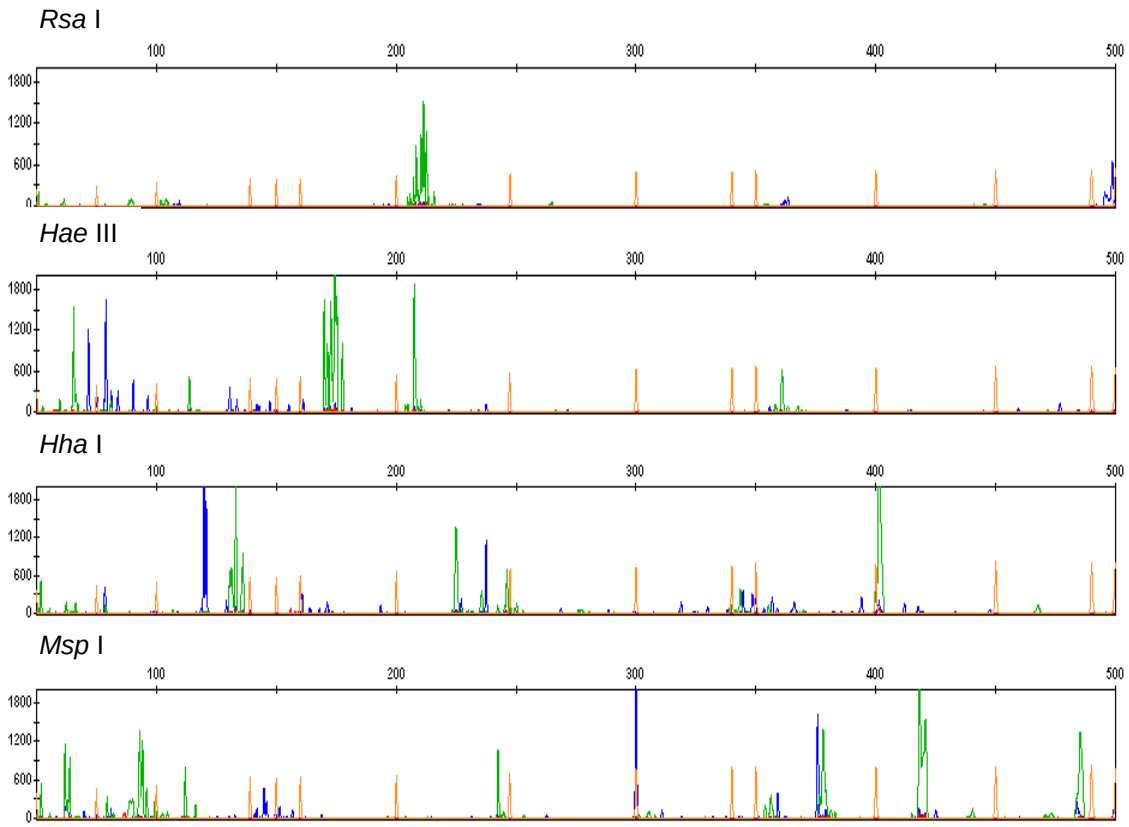

Figure 3.1-4: Electropherograms of T-RFs after digestion of multiplex PCR products with the restriction enzymes *Rsa* I, *Hae* III, *Hha* I and *Msp* I.
The blue peaks represent the terminal restriction fragments (T-RFs) of the PCR products with the 6-FAM fluorescent marker of the fungal primer. The green and red peaks are respectively the VIC (bacteria) and PET (*Rhizobium/Agrobacterium*) labels. The internal size standard is shown as the LIZ/orange dye. The T-RF position and height were estimated by reference to the internal standard. The vertical axis represents the peak height in relative fluorescence units (RFUs). The fragment sizes in base pairs (bp) are indicated on the horizontal axis.

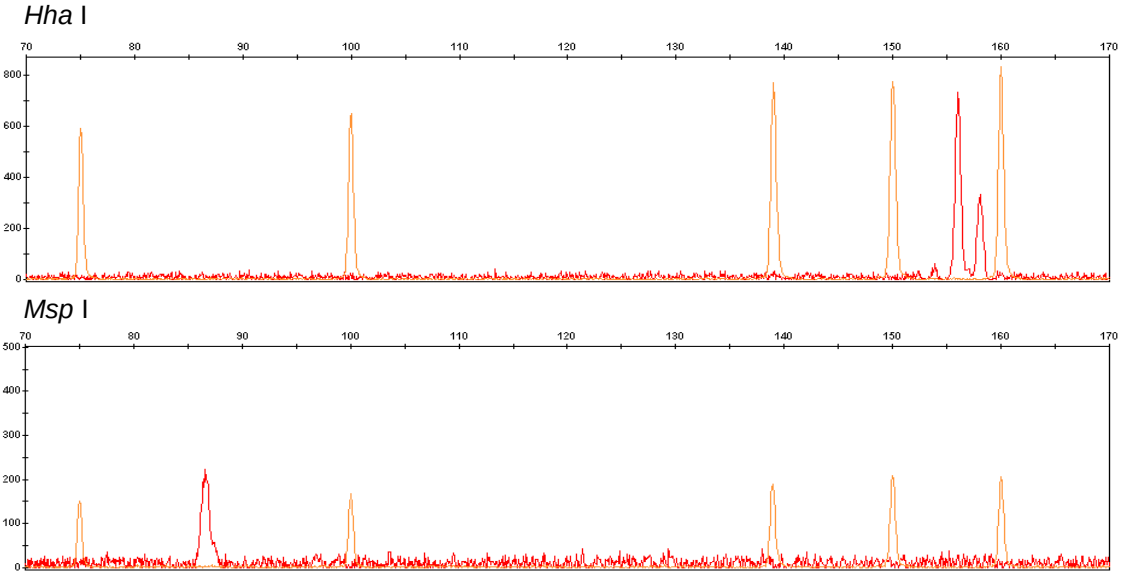

Figure 3.1-5: Electropherograms of T-RFs after digestion of individual PCR products of the RHIZ gene with the restriction enzymes *Hha* I and *Msp* I.
The graph on the top shows two representative peaks at 156 and 158 bp. The bottom graph has only one red peak at 87 bp.

3.1.2.2 Quantity of DNA for the digestion

Different concentrations of DNA were tested to evaluate the effect on the fragmentation pattern of the quantity of DNA digested.

T-RFLPs were performed by digesting 1000, 500 and 200 ng of DNA with *Hha* I only (data not shown). Digestion of 1000 ng of DNA produced a lot of peaks but most of them were too high compare to the size standard peaks, which resulted in an impaired estimation of the peak characteristics (position, height, area). Conversely, digestion of 200 ng of DNA produced peaks with very low height and many peaks could not be picked up by the GeneMapper software. Therefore, 500 ng of DNA was considered to be the best option, and was used for all further analyses.

3.2 Assessment of the methodology

In order to demonstrate the power of the association of the multiplex PCR with the T-RFLP analysis (multiplex T-RFLP), the reproducibility of the results obtained with the individual PCRs was contrasted with the results from the multiplex PCR.

The efficiency of the multiplex T-RFLP was assessed by comparing the fragmentation patterns of the multiplex PCR with the individual PCR and the mixed PCRs. This was performed for each gene with each enzyme.

3.2.1 16S and ITS genes

For these two markers, the fragmentation pattern was compared between the three approaches: individual PCR, mixed PCRs and multiplex PCR. One sample from each transect was randomly chosen, and for each enzyme, all the peaks present in each T-RFLP condition were processed according to the procedure detailed in the section 2.7.1.

As an example, Figure 3.2-1 to Figure 3.2-4 represent the T-RFLP profiles obtained on a random sample with each three T-RFLP approaches when using each enzyme on the genes 16S and ITS.

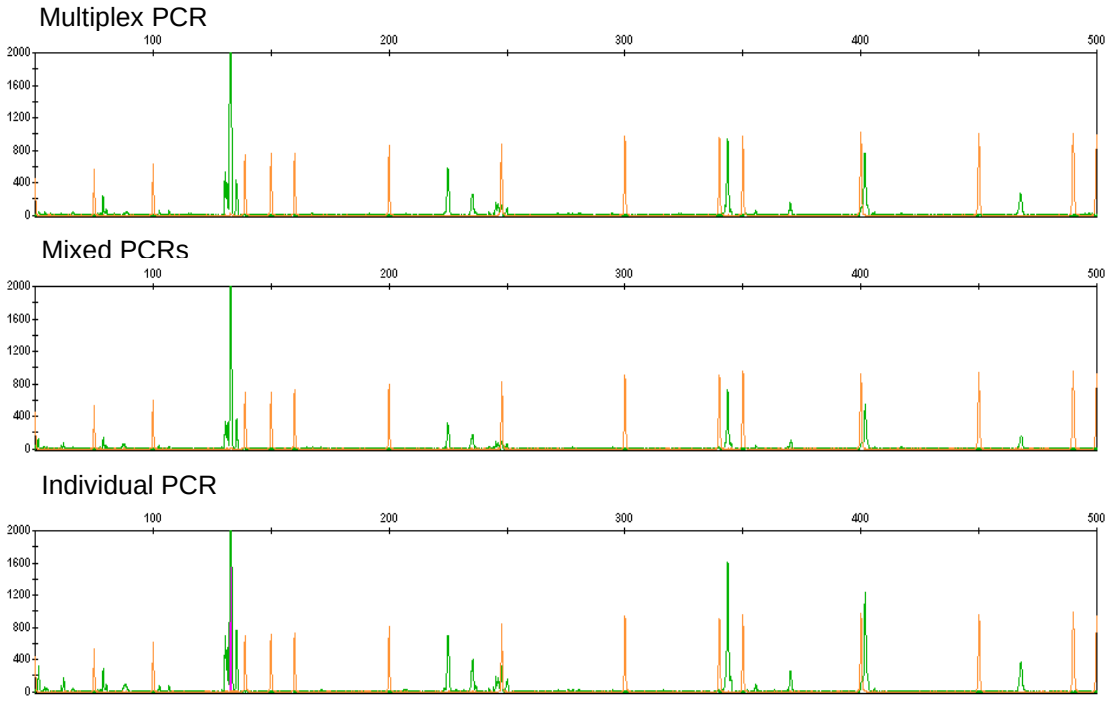

Figure 3.2-1: Electropherograms of the 16S T-RFs after digestion with the enzyme *Hha* I. Although the multiplex and mixed PCRs T-RFLP profiles contain all four fluorescent dyes, only the LIZ and VIC channels were switched on to help visualising the similarity of the profiles in the three conditions.

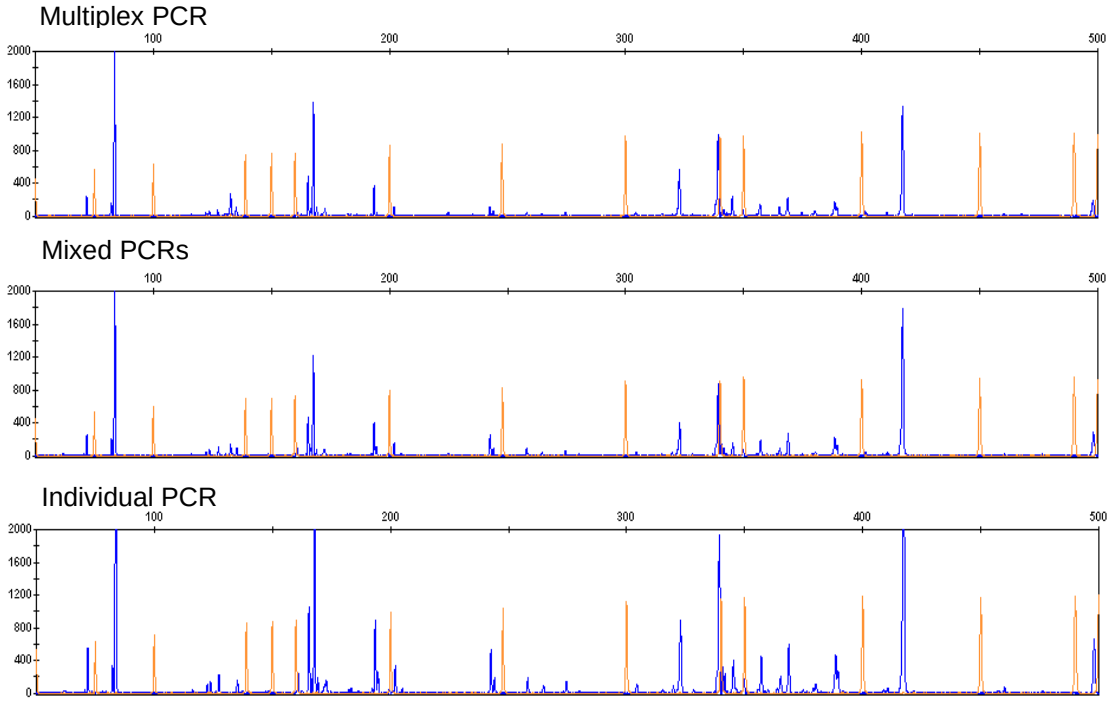

Figure 3.2-2: Electropherograms of the ITS T-RFs after digestion with the enzyme *Hha* I.

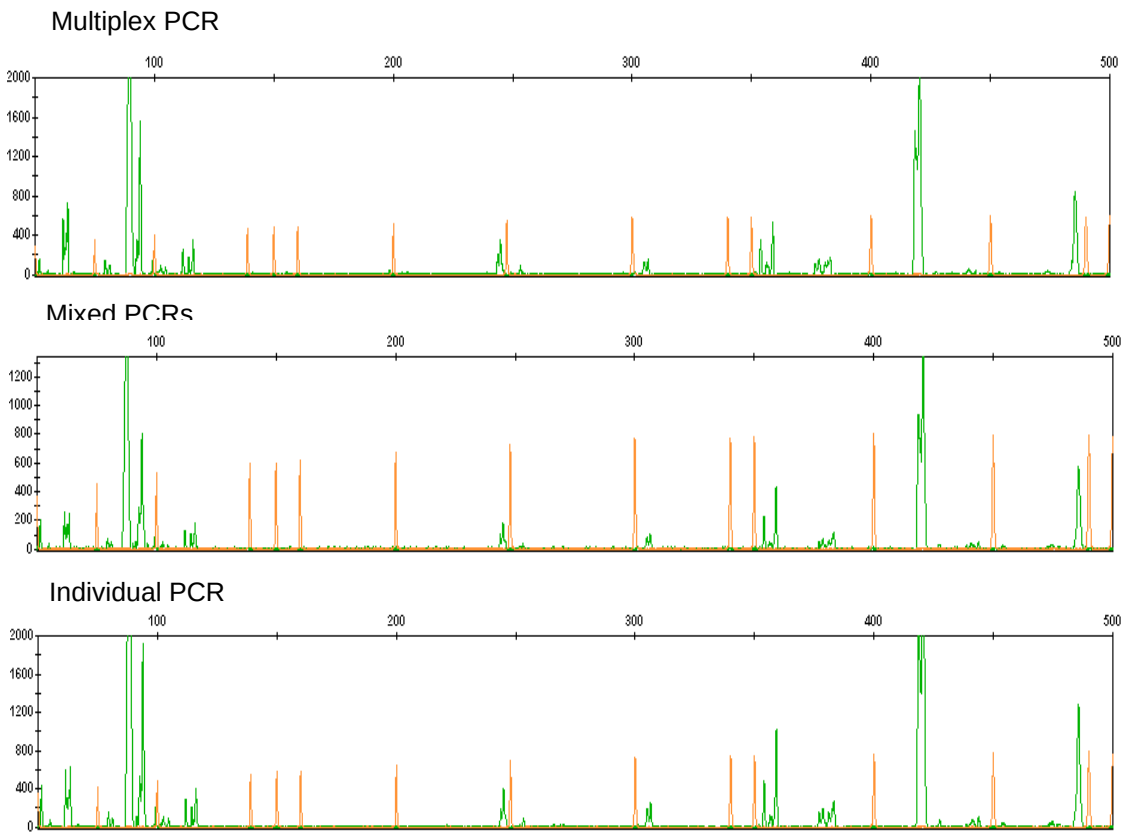

Figure 3.2-3: Electropherograms of the 16S T-RFs after digestion with the enzyme *Msp* I.

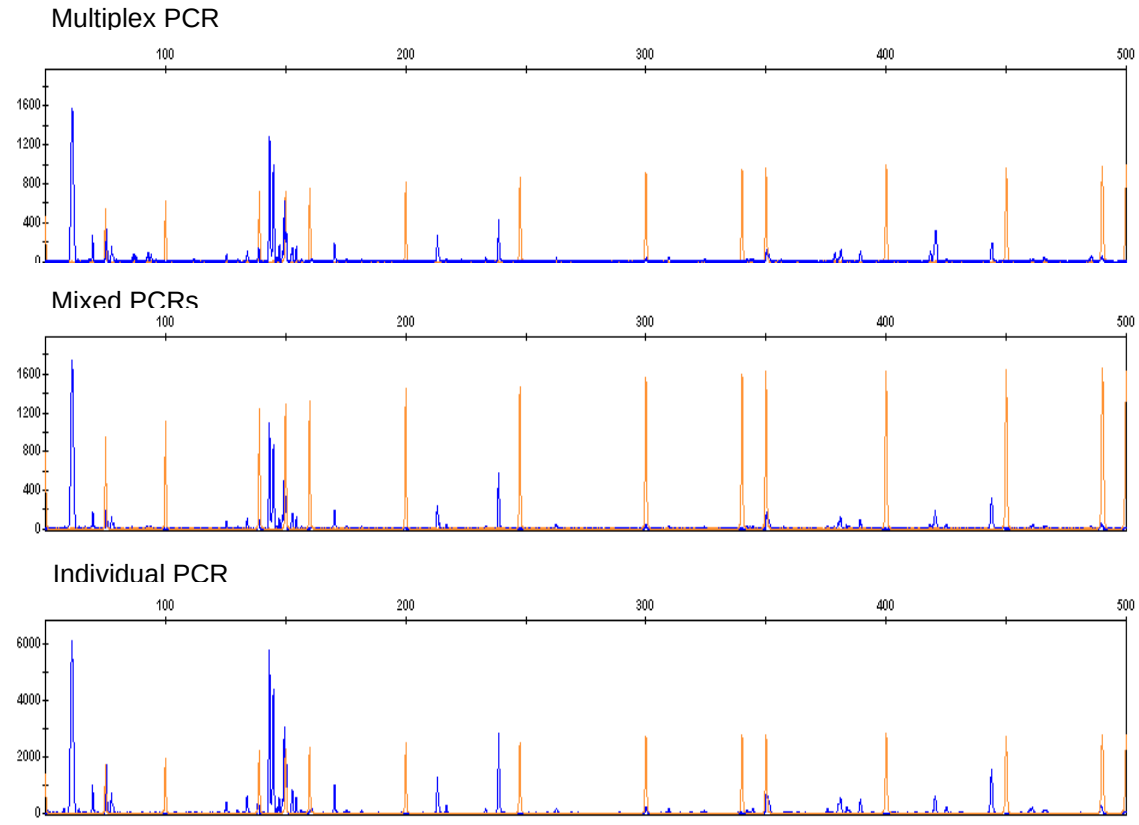

Figure 3.2-4: Electropherograms of the ITS T-RFs after digestion with the enzyme *Msp* I.

As shown in Figure 3.2-1 to Figure 3.2-4, the T-RFLP profiles generated by individual PCR and multiplex PCR were identical. The T-RFLP profiles produced by each enzyme all looked similar between the different conditions and for both the 16S and ITS genes.

The Table 3.2-1 shows the peaks/fragments of the 16S T-RFs detected in the three T-RFLP conditions for one sample only but with the two enzymes.

Fragment sizes	*Hha* I			Fragment sizes	*Msp* I		
	Individual PCR	Mixed PCRs	Multiplex PCR		Individual PCR	Mixed PCRs	Multiplex PCR
51	1	1	1	**52**	1	1	1
52	1	1	1	**61**	1	1	1
61	1	1	1	**62**	1	1	0
62	1	1	0	**63**	1	1	1
66	1	1	1	**64**	1	1	1
79	1	1	1	**79**	1	1	1
88	1	1	1	**88**	1	1	1
103	1	1	1	**90**	0	1	1
107	1	1	1	**93**	1	1	1
130	1	1	0	**94**	1	1	1
131	1	1	1	**99**	1	1	1
133	1	1	1	**104**	1	1	1
136	1	1	1	**112**	1	1	1
224	1	1	1	**116**	1	1	1
225	1	1	1	**240**	1	1	1
236	1	1	1	**245**	1	1	1
242	1	1	1	**305**	1	1	1
245	1	1	0	**354**	1	1	1
246	1	1	1	**356**	1	1	1
248	1	1	1	**359**	1	1	0
249	1	0	0	**377**	1	1	1
250	1	1	1	**379**	1	1	1
253	1	1	1	**382**	1	1	1
339	0	1	1	**418**	1	1	1
342	1	1	1	**420**	0	1	1
344	1	1	1	**421**	1	1	1
355	1	1	1	**441**	1	1	1
370	1	1	1	**486**	1	1	1
374	0	1	0	**Total fragments**	26	28	26
400	1	1	1				
402	1	1	1				
405	0	0	1				
468	1	1	1				
Total fragments	30	31	28				

Table 3.2-1: Fragments detected in a sample (*Vaccinum vitis-idaea*) from the forest transect (FV) after digestion of the 16S amplicons with each enzyme and for each approach.
The number “1” represents the presence of a fragment and the number “0” its absence.

Although the total number of fragments was different, similarities could be observed. For that reason, a similarity matrix was created to evaluate how many similar fragments were present in the different approaches.

The Table 3.2-2 summarises the percentage of similarity of the fragmentation pattern obtained between each T-RFLP condition with each enzyme for both 16S and ITS genes.

16S	***Hha* I**				***Msp* I**			
	FV	**FC**	**TC**	**MC**	**FV**	**FC**	**TC**	**MC**
Individual PCR Vs. Mixed PCRs	96.67	96.30	90.00	100.00	100.00	92.31	95.83	94.74
Individual PCR Vs. Multiplex PCR	92.86	96.00	100.00	91.30	92.31	96.15	84.62	94.74
Mixed PCRs Vs. Multiplex PCR	96.43	96.00	95.65	95.00	92.86	96.15	88.46	94.74

ITS	***Hha* I**				***Msp* I**			
	FV	**FC**	**TC**	**MC**	**FV**	**FC**	**TC**	**MC**
Individual PCR Vs. Mixed PCRs	96.77	100.00	91.67	100.00	96.30	86.67	95.00	91.30
Individual PCR Vs. Multiplex PCR	90.32	95.83	80.56	87.50	96.30	93.33	95.00	78.26
Mixed PCRs Vs. Multiplex PCR	90.32	95.83	86.11	95.83	93.94	100.00	89.29	77.78

Table 3.2-2: Similarity matrix of the different TRFLP conditions for each transect (16S and ITS). These values represent the analysis of only one sample but a similar trend was observed in the other samples.

The similarity between each approach was very close with percentages mostly over 90. Nevertheless, lower similarities (between individual and multiplex PCRs as well as between mixed and multiplex PCRs) were found in the moorland transect for the ITS gene digested with *Msp* I.

3.2.2 RHIZ gene

For this marker, very few peaks were expected. For that reason, the electropherogram was scanned only for the presence or absence of the predicted fragments in all 43 samples and for both enzymes.

The database search on the MiCA III web site gave a result of two terminal fragments of 153 and 154 bp with *Hha* I and one peak of 90 bp with *Msp* I.

3.2.2.1 Digestion with *Hha* I

As an example, the Figure 3.2-5 represents the T-RFLP profiles obtained on a random sample with each three approaches when using the enzyme *Hha* I on the RHIZ gene.

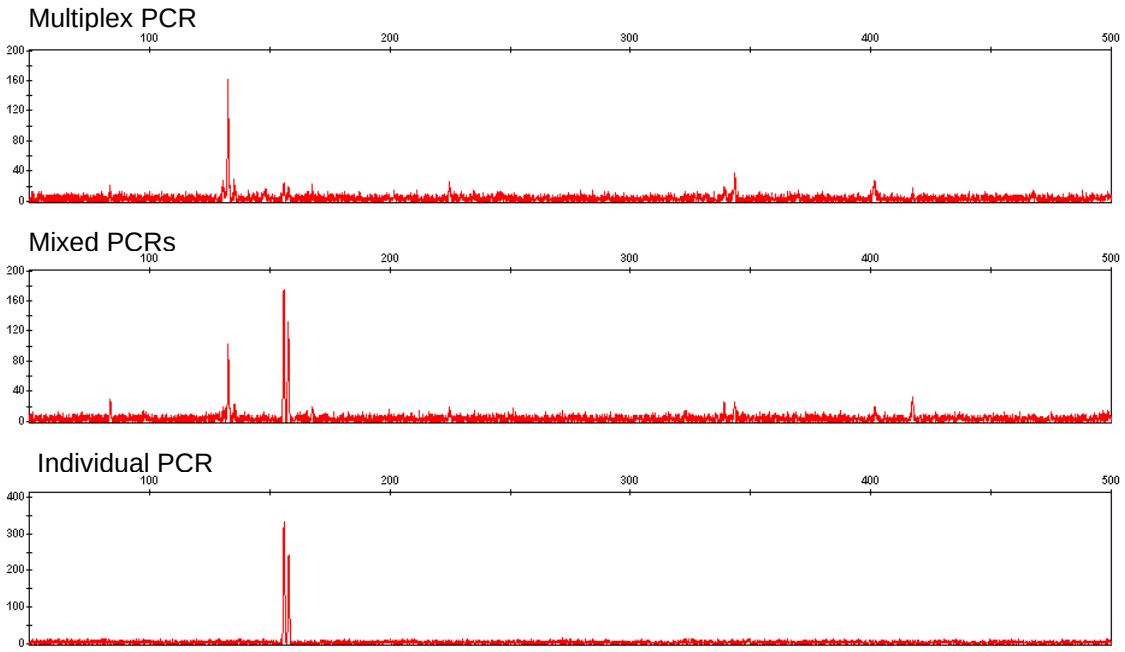

Figure 3.2-5: Electropherograms of the RHIZ T-RFs after digestion with the enzyme *Hha* I.

The three electropherograms showed a similar profile. The two predicted peaks were not observed at the expected size. Instead, two other fragments (156 and 158 bp) were observed in the individual PCR but their intensity decreased greatly in the multiplex PCR. The mixed and multiplex PCRs shared similar unexpected peaks at 133, 225, 343 and 401 bp.

The presence and absence of the expected peaks was analysed in all 43 samples (data not shown). Although the similarities between individual and mixed PCRs were very high, very few samples contained the expected fragments in the multiplex PCR.

As a summary, the Table 3.2-3 gives the similarities of the percentage of samples in which either one or both fragments were present.

***Hha* I**	**Both fragments present**	**One fragment present**
Individual PCR Vs. Mixed PCRs	93.02	97.67
Individual PCR Vs. Multiplex PCR	9.30	30.23
Mixed PCRs Vs. Multiplex PCR	10.00	30.95

Table 3.2-3: Percentage of similarity between the different T-RFLP approaches for the RHIZ gene digested with *Hha* I.

The similarity between the T-RFLP methodologies was higher when considering the presence of one fragment only.

3.2.2.2 Digestion with *Msp* I

As an example, the Figure 3.2-6 represents the T-RFLP profiles obtained on a random sample with each three approaches when using the enzyme *Msp* I on the RHIZ gene.

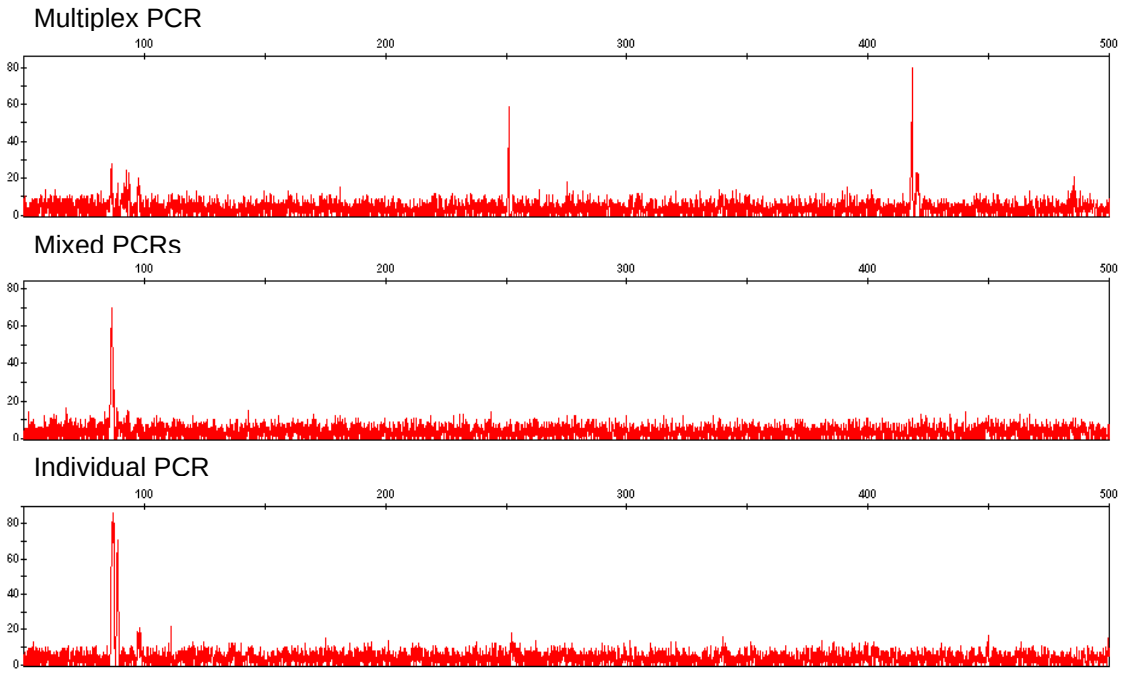

Figure 3.2-6: Electropherograms of the RHIZ T-RFs after digestion with the enzyme *Msp* I.

The three electropherograms showed a similar profile. Two peaks (87 and 89 bp) were present in the individual PCR but their intensity decreased greatly in the multiplex PCR. Unexpected peaks at 251 and 418 bp were observed in the multiplex PCR condition.

In all samples from the individual PCRs, two peaks were observed at 87 and 89 bp where only one peak was expected at 90 bp. In the samples from the mixed and multiplex PCRs, many fragments of 86 and 88 bp were present. Therefore, a 3-bp window (87-89 bp) was chosen in order to gather as many peaks as possible and manual alignment of overlapping peaks (mainly at 86 bp) was necessary in order to obtain more consistent results. This is a well-established procedure for assigning identification of a particular T-RFLP peak.

The presence and absence of the peaks was analysed in all 43 samples (data not shown). Although the results in the individual PCR and mixed PCRs looked similar, the multiplex PCR gave a wider range of fragmentation within the 3-bp window.
As a summary, the Table 3.2-4 indicates the similarities of the percentage of samples in which at least one fragment was present between 87 and 89 bp.

***Msp* I**	**Fragments present in the window 87-89 bp**
Individual PCR/Mixed PCRs	100.00
Individual PCR/Multiplex PCR	85.71
Mixed PCRs/Multiplex PCR	85.71

Table 3.2-4: Percentage of similarity between the different methodologies for the RHIZ gene digested with *Msp* I.

The fragmentation pattern was highly similar in all methodologies.

3.3 Impact of plant species and habitat on the microbial community

The binary data collected from all 43 samples were analysed using the principal co-ordinate (PCO) function of multivariate analysis to identify underlying patterns. The three T-RFLP approaches (individual PCR, multiplex PCR, mixed PCRs) were analysed separately and compared with each other for the bacterial and fungal markers and for the two enzymes used.

The Figure 3.3-1 shows the clustering of samples according to the PCO scores of dimensions one (PCO-1) and two (PCO-2). These PCO scores were obtained from the analysis of the 16S marker digested with the enzyme *Hha* I.

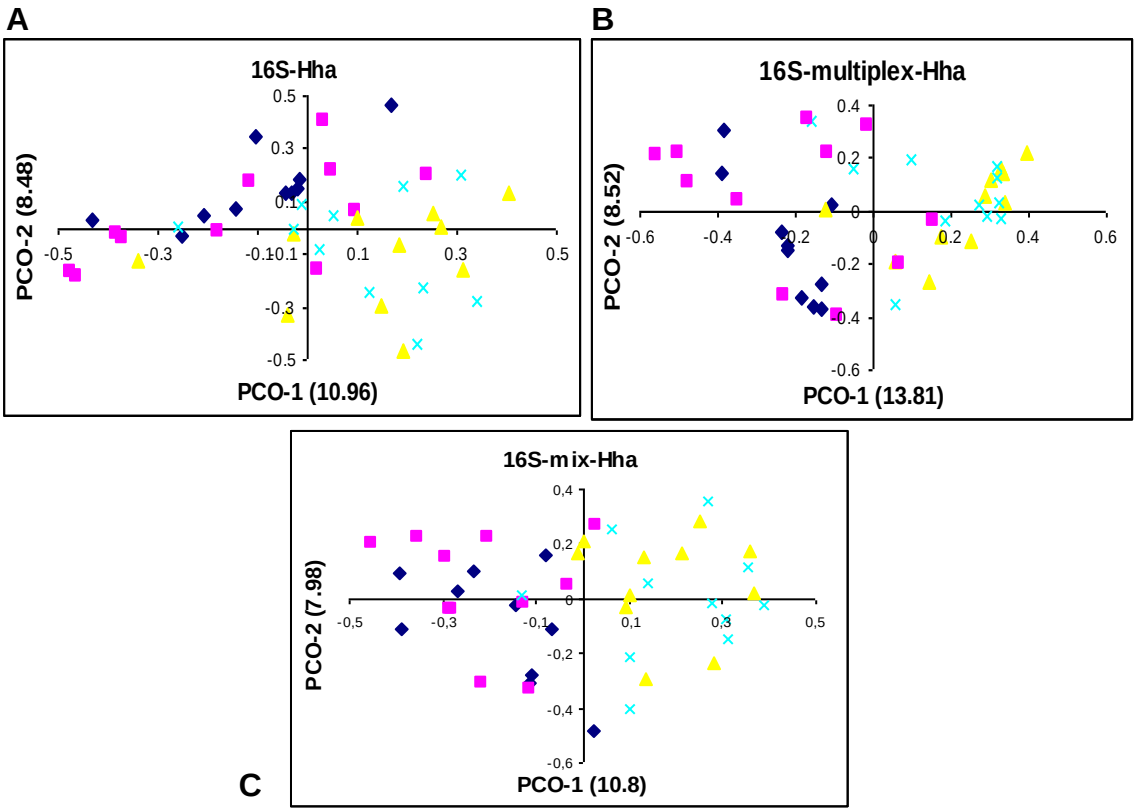

Figure 3.3-1: Coordination diagrams of the 16S gene analysed with the three T-RFLP approaches after digestion by *Hha* I.
A = individual PCR; B = multiplex PCR; C = mixed PCRs.
In brackets are indicated the percentage of variation of each dimension of the PCO.
Plot legend: diamond = FV, square = FC, triangle = TC, cross = MC.

Results from the PCO analysis of the 16S marker digested with the enzyme *Hha* I showed that the first three dimensions of PCO accounted for a typical 27 % of the variability. Further analysis resulted into a clustering of the samples taken from the forest (FV and FC) and the samples from the moorland and transition (MC and TC). This pattern was consistent in all three T-RFLP approaches.

The Figure 3.3-2 shows the clustering of the PCO scores obtained from the analysis of the ITS marker digested with the enzyme *Hha* I.

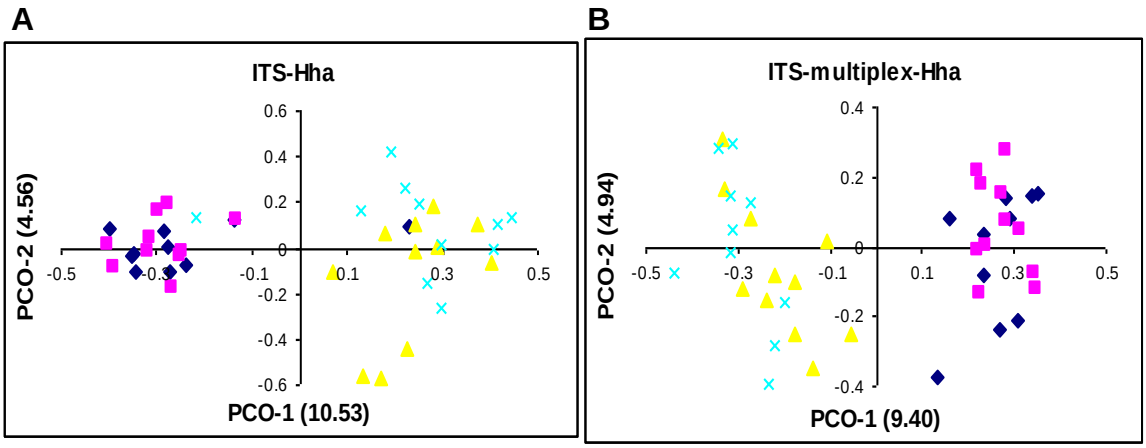

Figure 3.3-2: Coordination diagrams of the ITS gene analysed with the individual (A) and multiplex (B) PCRs after digestion by *Hha* I.
In brackets are indicated the percentage of variation of each dimension of the PCO.
Plot legend: diamond = FV, square = FC, triangle = TC, cross = MC.

Results from the PCO analysis of the ITS gene digested with the enzyme *Hha* I were similar to the 16S data (Figure 3.3-1) but with a stronger clustering of the soil types where all the samples from the forest, irrespective of the plant type, were clustered together. Similarly, the samples from the moorland and the transition habitats were in the same cluster. This pattern was consistent in the individual PCR (A) and the multiplex PCR (B). The first three dimensions accounted for about 19 % of the total variance.

The Figure 3.3-3 shows the clustering of the PCO scores obtained from the analysis of the 16S and ITS markers digested with the enzyme *Msp* I after individual PCR.

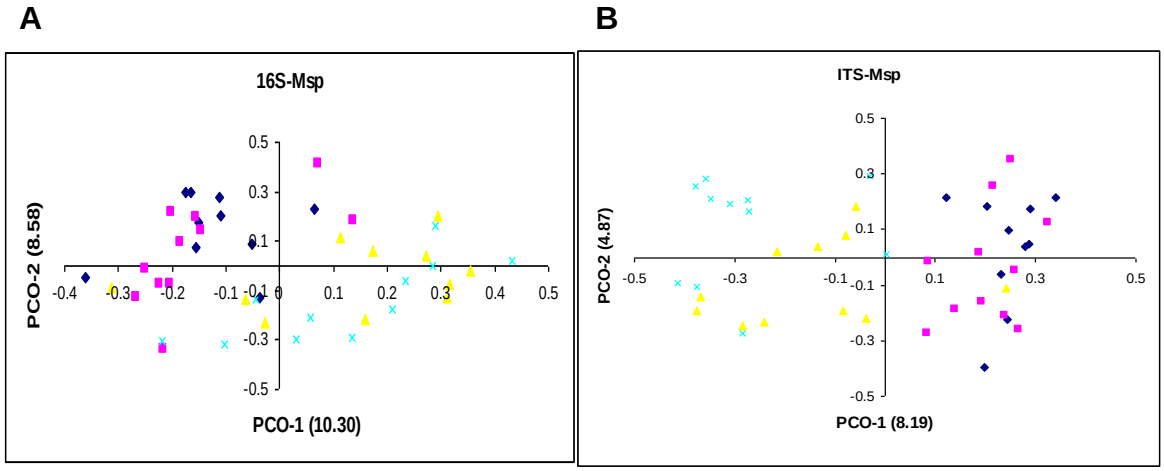

Figure 3.3-3: Coordination diagrams of the 16S (A) and ITS (B) genes analysed with the individual PCR after digestion by *Msp* I.

As observed with *Hha* I, digestion with *Msp* I resulted in a clustering of the samples following a habitat-dependant pattern, irrespective of the plant species. Again, the separation between the soil types was stronger for the ITS gene. A variability of about 27 % (16S) and 17 % (ITS) for the first three dimensions was observed.

Chapter 4
Discussion

This study was designed to develop a novel molecular technique, the multiplex T-RFLP, based on the combination of two particular methods: multiplex PCR and T-RFLP. Its applicability as a novel DNA fingerprinting method to study the soil microbial diversity and structure was also evaluated in an environmental gradient such as a forest-moorland transition.

Only a few studies have been carried out where both bacterial and fungal communities were studied to examine biotic interactions. In all such studies, analysis for individual species was carried out separately using DNA fingerprinting methods (Nunan *et al.*, 2005; Anderson *et al.*, 2003). This approach is therefore time-consuming and expensive. Both communities could be analysed at the same time using the multiplex PCR technique that allows amplifying several gene markers at once.

4.1 Optimisation of the multiplex T-RFLP

In a first step, our primary results of individual PCRs showed it was possible to use the same PCR program to amplify the three genes under investigation; the bacterial 16S rDNA, the fungal-specific ITS region of the 18S rRNA gene and the 16S rDNA specific to *Rhizobium/Agrobacterium* genera (Figure 3.1-1). Nevertheless, modifications of the PCR conditions had to be made such as a two-fold increase of the concentration of the fungal primer (Figure 3.1-2) due to a lower fungal genome number. It allowed increased amplification of fungal DNA without having to increase the number of cycles, which would have increased the total amount of DNA amplified in the multiplex PCR.

Finally, the multiplex PCR was assessed by comparing the band migration of multiplex PCR products on an agarose gel with the bands of amplicons from individual PCRs. The band pattern observed in the multiplex PCR was a mixture of the band migration pattern of each individual PCR (Figure 3.1-3). The bacterial 16S and fungal ITS bands were clearly identified although the rhizobial/agrobacterial 16S (RHIZ) was not observed due to the proximity and brightness of the 16S band. However, the gene was amplified because terminal restriction fragments (T-RFs) were later analysed in the T-RFLP mixture after enzymatic digestion of the PCR products.

The multiplex PCR was used successfully to amplify genes of different microbial communities at the same time. This technique has been widely used, in particular to simultaneously identify different pathogenic organisms (Way *et al.*, 1993). Our study confirmed the multiplex PCR can be successfully used for amplification of DNA from environmental samples.

In a second step, T-RFLP analysis was optimised through the choice of the enzymes used for the digestion of the PCR products. The restriction enzymes tested were *Hae* III, *Hha* I, *Msp* I and *Rsa* I. It was important to choose enzymes that give a good fragmentation pattern because the number of restriction fragments generated is an indicator of the microbial diversity since each fragment size observed is supposed to be specific to a microbial genome (Liu *et al.*, 1997). *Hae* III and *Rsa* I did not show a consistent pattern and even distribution, whereas *Hha* I and *Msp* I generated a wide range of peaks for the 16S and ITS genes (Figure 3.1-4). The number and quality of the peaks generated were especially important for the detection of the RHIZ gene because microorganisms belonging to the *Rhizobium/Agrobacterium* genus are not abundant in some soils. Where the enzymes *Rsa* I and *Hae* III produced respectively none and one (174 bp) peak, the *Msp* I and *Hha* I digestions showed to be more efficient by correspondingly generating one (87 bp) and two (156 and 158 bp) peaks (Figure 3.1-5). The size of these fragments was compared with a web-based search on the Mica III website (http://mica.ibest.uidaho.edu/trflp.php), which gave two fragment sizes of 153 and 154 bp for the digestion with *Hha* I and one fragment of 90 bp for the digestion with *Msp* I. These values are slightly different from our experimental

values. The database search on MiCA III is performed according to the information given on the webpage, *i.e.* the primer sequences and the restriction enzyme used. The results found correspond to fragment sizes estimated by previous workers using their own technique/protocol. Therefore, it is expected to observe differences with experimental values. It could be primarily due to the type of column and polymer used for the separation of the restriction fragments. This observation is consistent with many previous works (Liu *et al.*, 1997; Christensen *et al.*, 2003) in which a few base pair discrepancy was always found between the experimental and predicted fragment sizes.

Finally, several quantities of DNA were digested to observe the effect on the fragment analysis. Using a low quantity of DNA (200 ng) gave fewer peaks, which means the less abundant fragments were not picked up by the detection software. In contrast, a high DNA quantity (1000 ng) was not the best due to the most abundant fragments (high peaks) being off-scale. On the other hand, the less abundant fragments were bettered visualised. As a compromise, 500 ng of DNA was used for the digestion step.

4.2 Assessment of the T-RFLP

The consistency of the multiplex T-RFLP was compared to T-RFLPs performed on individual and mixed PCRs. Each gene marker under investigation was analysed after digestion with each enzyme.

When analysing the results of the 16S and ITS genes, the T-RFLP profiles from each enzyme used and from each approach looked similar (Figure 3.2-1 to Figure 3.2-4). The similarity matrix (Table 3.2-2) emphasised on the efficacy of the multiplex T-RFLP to produce reliable results since the percentages of similarities between the three approaches were mostly above 90 %. Similarity was observed in all transects although values close to 80 % were obtained for the transition and moorland transects of *Calluna vulgaris* when the ITS gene was digested with *Hha* I or *Msp* I. However, a close look into individual profiles suggested that similarity matrix would be near 100 % for each and every sample if we had analysed all the fragments generated. But we decided to

discard fragments which had fluorescence intensities less than 0.5 % of the total fluorescence. This is a well established approach to discard non-representative fragments (Blackwood *et al.*, 2003).

The results of the study of the RHIZ gene with multiplex T-RFLP gave different similarities depending on which restriction enzyme was used. *Hha* I did not give a good reproducibility since only 30 % of similarity was found between the T-RFLP profiles from the individual and multiplex PCRs and when considering the generation of only one fragment where two were expected (Table 3.2-3). Barely 9 % of the total samples had the two fragments present. This was an unexpected result and was probably due to a lack of sufficient RHIZ amplicons. Besides, it could be related to the fact that with multiplex T-RFLP, the fluorescence intensities (*i.e.* the peak heights) of the fragments detected were always lower compared to the corresponding fluorescence observed from the individual PCRs. The peak detection was run again with an intensity threshold set at 5 RFUs as compared to 20. The two expected fragments were present in all the samples. Thus, the efficiency of the enzyme could not be in cause. The problem was at the detection level. For an unexplained reason, the detection of the rhizobial 16S was rendered difficult in the multiplex condition.

Finally, the detection of the RHIZ product terminal fragment gave a good similarity of 86 % between the T-RFLP profiles from the individual and multiplex PCRs for samples digested with *Msp* I (Table 3.2-4). Results were improved by the use of a 3-bp window that contained the two most abundant fragments (87 and 89 bp) because only one fragment at time was predicted by the MiCA III webpage. Using a window for overlapping peaks is a common thing to do and was proven to be useful in the determination of the size of fragments generated with the enzyme Msp I (Christensen *et al.*, 2003).

The observation of unexpected peaks (133, 225, 343 and 401 bp with *Hha* I; 251 and 418 bp with *Msp* I) in the mixed and multiplex PCR approaches was inconsistent with what was observed in the T-RFLP profiles from the individual PCRs. These fragments could be artefacts or incompletely digested fragments but their occurrence does not change the quality of the results because only the presence or absence in the samples of the expected fragments from the digestion of the RHIZ sequence was evaluated.

4.3 Improvement of the methodology

Multiplex T-RFLP proved to be working although the detection of the T-RFs from the digestion of the rhizobial/agrobacterial marker showed a lack of detection efficiency. This could be improved at several stages.

First of all, the specificity of the rhizobial primers could be improved. Indeed, the rhizobial reverse primer (1244r) used produces an amplicon which has an annealing site for the universal bacterial primer (1087r) and this reduces the number of RHIZ amplicons in every cycle. This problem will be tackled later when the 1492r primer will be used instead or 1087r.

Second of all, it is widely known that the fluorescence intensity of the 6-FAM molecule is higher than some other dyes used for the T-RFLP. For that reason, Christensen and colleagues (2003, 2004) used a 1:4 ratio of the 6-FAM labelled primer. In our study, increasing the concentration of the PET labelled primer for the amplification of the RHIZ gene may help to yield the detection sensitivity of the rhizobial T-RFs for the multiplex T-RFLP.

Overall, the improvement of these two parameters should greatly enhance the quality and intensity of the rhizobial/agrobacterial peaks detected by multiplex T-RFLP.

A T-RFLP pattern produced from amplified 16S rDNA is a simplified representation of a microbial community. Different species, regardless of taxon, can share the same T-RFs from a given restriction enzyme, which results in a less accurate community characterisation. For that reason, it is recommended to use combined restriction patterns from different enzyme digests (Clement *et al.*, 1998).

4.4 Impact of plant species and habitat on the microbial community

The PCO analyses on the bacterial and fungal sequences (Figure 3.3-1 to Figure 3.3-3) showed that the soil type (*i.e.* the transect where the samples were from) had an influence on the fragmentation pattern. This result was observed with the two enzymes. Since the restriction fragments produced are specific to the microbial species, this means the soil type influences the microbial diversity. This effect was independent of the plant species. This is in contradiction with other authors who found the plant species to be the factor influencing the change of microbial diversity (Grayston *et al.*, 1998). Their results were based on the analysis of different soils using the Biolog® method, which was proven not to be sensitive enough since it does not take into consideration the uncultivable species (Singh *et al.*, 2005). Our results agree with other studies performed on grassland fields and using similar molecular fingerprinting techniques such as DGGE and T-RFLP (Nunan *et al.*, 2005; Singh *et al.*, 2005). The difference in the microbial community composition between the soil types was stronger for the fungal species (Figure 3.3-2). Similar results were found in the past from DGGE analyses (Anderson *et al.*, 2003).

4.5 Future directions

Multiplex T-RFLP proved to be efficient and reliable in detecting the changes in microbial community diversity. The next step would be to characterise the community structure associated to each soil type/plant species. It can be performed by sequencing the soil DNA or by comparing the T-RFLP profiles to a web-based database. While the fungal database is under-developed, bacterial species can be searched through databases such as MiCA III or the Ribosomal Database Project II (RDP-II) (http://rdp.cme.msu.edu/). T-RFLP

profiles can be compared and corresponding organisms can then be identified. The RDP-II tool was used in association with multiplex T-RFLP by Christensen and colleagues (2003, 2004) to set up a protocol for the rapid detection of Bacteria in positive blood cultures. They concluded this was a reliable approach and application to non-bacterial organisms should be attempted.

Other applications include the novel technique of real time T-RFLP, which incorporates the quantitative feature of the real time PCR and the fingerprinting feature of the T-RFLP analysis. This allows simultaneous determination of microbial diversity and abundance within a complex microbial community (Yu *et al.*, 2005). Their results showed the utility of a tool such as real-time T-RFLP: while T-RFLP profiles displayed the presence of the same microorganisms between different samples, the real-time data gave information on the abundance of specific T-RFs and thus, on the dominant microbial function. Obtaining such information could be extremely useful in ecological microbiology, especially to evaluate how a given microbial community structure can influence its ecological niche. Although the real-time T-RFLP assay was applied to environmental samples (wastewater) as well as enrichment cultures and also to detect pure bacterial strains in a model community, it could be easily applied to various natural habitats such as soils.

In conclusion, our study proved multiplex T-RFLP is a very powerful technique for the detection of changes within a soil microbial community. The use of multiplex PCR to amplify several microbial genera at once for their simultaneous detection is a significantly time and cost-saving procedure. The applications of this technique to microbial ecology studies are wide. This technique can be used in study on interactions between different biotic groups, impact of introduced organisms on soil and water microbial community, investigating soil and water microbial indicators, links between structural and functional diversity, *etc*. Although only structural genes were tested and improvements are still needed for the detection of specific groups (*e.g. Rhizobium/Agrobacterium*), functional genes can be analysed to go deeper into the understanding of biotic interactions in the rhizosphere and other ecosystems.

Chapter 5
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